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Microencapsulation of hepatic cells for extracorporeal liver supply

Vittoria Pandolfi

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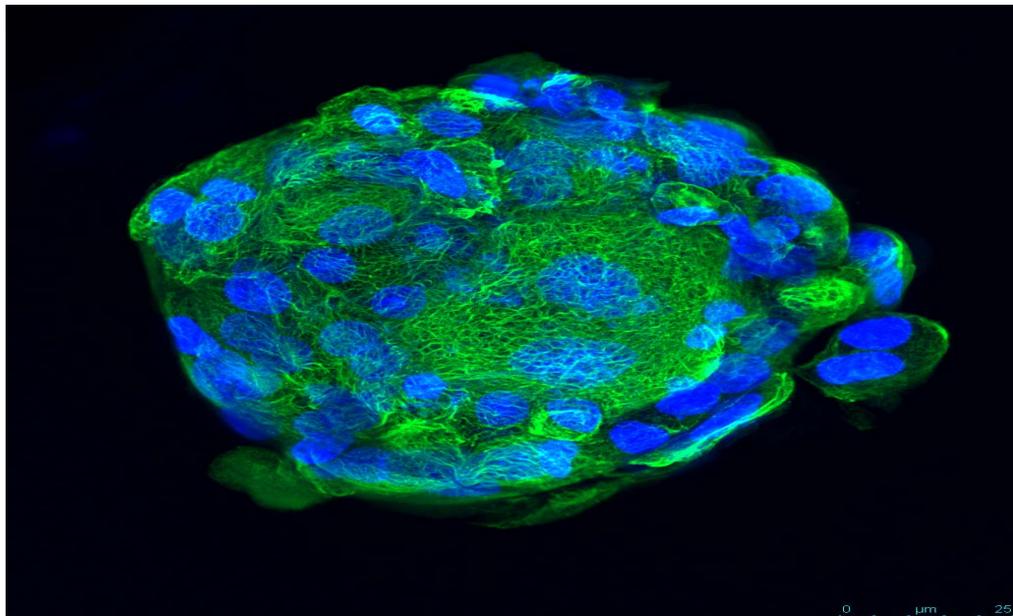
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Par Vittoria PANDOLFI

Microencapsulation of hepatic cells for extracorporeal liver supply

Thèse présentée
pour l'obtention du grade
de Docteur de l'UTC



Soutenue le 17 mars 2016
Spécialité : Biomécanique et Bio-ingénierie

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Laboratoire : Biomécanique et Bioingénierie

PANDOLFI Vittoria

**MICROENCAPSULATION OF HEPATIC CELLS FOR
EXTRACORPOREAL LIVER SUPPLY**

Soutenue le 17 mars 2016

Thèse dirigée par Cécile LEGALLAIS

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*“There is no end. There is no beginning.
There is only the infinite passion of life”
- Federico Fellini -
Italian film director (1920-1993)*

To my family

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GENERAL INTRODUCTION

Every year, between one to six cases of *acute liver failure* (ALF) per million people occur in the developed countries. ALF, although theoretically reversible, is a life-threatening illness which entails sudden loss of functions in a previously normal liver and is associated with high mortality (40-50%) in the most severe cases. Several causes have been identified, such as hepatitis and drug overdoses (e.g. paracetamol), which differently predominate worldwide. Liver transplantation remains the only therapeutic solution determining survival in patients with irreversible AFL. The major limitation of this definitive treatment is the shortage in transplantable organs, which, as consequence, permanently increases the number of patients on waiting list. The need of substitutive treatments remains thus urgent and fundamental.

To complete the offer of purely artificial liver such as MARS® or Prometheus® systems, *bioartificial liver (BAL) assistant devices* represent a valuable temporary alternative for the future. Both system categories intend to bridge patients till their liver regenerates or undergoes transplantation. During waiting time, BAL treatment could not only offer blood purification and detoxification (from protein bound and water soluble substances) by means of albumin dialysis, plasma separation and filtration, and/or plasma exchange, but also substitute specific liver metabolic functions through the activity of a cellular component - the hepatocytes - integrated in the system and, more specifically, housed in acclimating containers, so-called *bioreactors*. Pre-clinical and clinical trials have already established a proof of concept, but the promising role of the BAL devices in supplying patients in acute/fulminant liver failure still need to be demonstrated: none of the tested design evidenced significant effect of the BAL treatment on patient survival, may be because of the patients' typology, but also because of limited efficiency of the BAL components.

Primary human hepatocytes (PHH) are the ideal cell type to use in BAL devices. The definition of a suitable and sufficient mass of hepatocytes (minimum 20% of liver mass corresponding to about 200 g or 20×10^9 hepatocytes) is a fundamental parameter for the adequate replacement of the hepatic functions and, consequently, the correct performance of these systems. However, this aspect represents one of the main constraints to the exploitation of BAL devices in clinical applications. In fact, similarly to the shortage of transplantable livers, PHH are scarcely available and often do not show optimal functionalities, as they are especially sorted from discarded donor livers. In addition, PHH, but more in general primary hepatocytes, are sensitive cells that rapidly dedifferentiate and lose their specific functions when they are cultivated in *in vitro* environments. To overcome these obstacles, two solution lines can be broadly distinguished: i) seeking other cell sources may solve the issue of poor PHH availability, but it may be limited by other constraints, such as regulatory aspects, for cell lines or animal hepatocytes, or lack of maturation (up to now) and ethical issues, for pluripotent stem cells; ii) re-creating more physiological culture microenvironments may maximize and preserve PHH functionalities. In this latter context, several elements have been identified to be beneficial to PHH functional maintenance.

On the one hand, re-establishment of an *in vitro* three-dimensional configuration, accomplished via introduction of *ECM components* and/or cell self-assembly into aggregates (e.g. *spheroids*), favored longer survival of the hepatocytes as well as manifestation of extended liver specific functions and cyto-architectural similarities to the *in vivo* cellular configuration.

On the other hand, *co-culture* of hepatocytes with supporting cells has been pointed out as the most promising strategy for stabilizing and preserving hepatocyte behaviors over time in

in vitro conditions. Assisting cell types, in fact, hold their ability to influence functional responses of the hepatocytes by providing them with cues of the native organ. Multiple factors, such as direct (homotypic HEP interactions, heterotypic interface, and homotypic interplays of the nonhepatocyte populations) and indirect (soluble signaling molecules or insoluble ECM components) variables, have been considered to be responsible for the modulation and improvement of hepatocellular functionality in co-cultures. Beneficial contribution of the supporting cells has been largely investigated in numerous two- and three-dimensional co-culture models, generally, through measurements of albumin production, urea secretion, and cytochrome P450 activity of the hepatocytes. Most studies, though, were based on simplified co-culture platforms, which included only one supplemental cell population to the hepatocytes and, therefore, lacked the intricate cellular component of the liver. In alternative models, hepatocytes were co-cultured with the whole liver non-parenchymal cell fraction. In this configuration, composition of the non-parenchymal fraction was frequently overlooked and seeding densities were less controllable, omitting, thereby, key cellular proportions with which cells compose the liver (i.e. 60% of hepatocytes and 40% of non-parenchymal cells). Only recent works took in consideration both cellular complexity of the original organ and control on cell seeding densities. However, most co-culture settings were designed on the use of cell lines or two-dimensional (2D) pre-acclimated primary cells (namely, subject to initial 2D culture periods before their co-culture involvement). In this manner, included assisting cells may not possess those features that are unique to the non-hepatocyte cells of the liver and responsible for certain specific orchestrated interplays that occur between hepatic cells in *in vivo* situations.

The scope of this research work has been to propose a novel approach of co-culture which, by fulfilling the gaps left from previous studies, may sustain hepatocytes in the performance of their functionalities in the environment of the fluidized bed bioreactor (designed in our laboratory) in future investigations.

Definition of this co-culture model took inspiration from the intralobular structure of the liver that is constituted by one-cell-thick plates of hepatocytes spaced by hepatic sinusoids. These latter, which are peculiar blood capillaries of the organ, are formed by liver sinusoidal endothelial cells and inhabited by Kupffer cells and hepatic stellate cells. These three sinusoidal non-parenchymal cell populations, organized in a three-dimensional (3D) configuration according to specific relative proportions, primarily cooperate with the hepatocytes for the correct performance of liver's duties. Accordingly, the co-culture model resulted from the combination of paramount aspects: 1) presence of multiple cellular components, 2) cell arrangement in 3D structures, and 3) establishment of cell proportions. It was, thereby, composed of fresh isolated liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells in addition to hepatocytes cultured according to defined ratio under the shape of spheroids. The resulting system was defined *spheroidal multi-cultured model*. Its accomplishment required several steps which are the subject of the following chapters. In brief, these consisted in the:

1. Establishment of a protocol for the simultaneous isolation of the four cell populations from the same liver (CHAPTER 2).

Considering the shortage of human hepatic cells, the rat was selected as cell source in this work in order to assure the availability of the cellular component for the definition of the co-culture construct. If cells from different species are considered, future exploitation of the model should lead to appropriate adjustments.

2. Selection of culture parameters (medium composition and cell ratio) for optimal co-culture conditions (CHAPTER 3).

3. Subsequent characterization of the resulting model in terms of cellular organization and functional assessment (CHAPTER 4).

The spheroidal multi-cultured model was finally employed in the microenvironment of alginate beads (CHAPTER 5), which represent the immunological and mechanical barrier of the bioactive component in the fluidized bed bioreactor. Functional evaluation was carried out in this context.

CHAPTER 1
BACKGROUND AND RESEARCH CONTEXT

"Now, why is the stomach surrounded by the liver? Is it in order that the liver may warm it and it may in turn warm the food? This is indeed the very reason why it is closely clasped by the lobes of the liver, as if by fingers." – Galen, ca. 200 A.D.

1. OVERVIEW OF THE HEALTHY LIVER

The liver is one of the most intriguing organs of the body which, working “behind the scenes”, carries out more than 300 functions and supports the correct performance of other organs’ duties. Its mysterious entity has fascinated lots of researchers at different periods of time. In antiquity, liver was considered as one of the three fundamental organs of the body. For more than 1000 years, consequently to the theories of the Greek physician Galen (ca. 200 A.D.), this organ was considered to be the factory of the blood, produced by transforming the food after its collection from the gut, and the source of the veins. The liver was also regarded as the seat of the soul, identified as the center of affections and emotions. Controversially, it was referred as origin of a large number of diseases. By the mid-seventeenth century, thanks to increased numbers of animal vivisections and resulting discoveries, the Galen’s doctrines were questioned. The main role ascribed to the liver until that time was denied from Bartholin (1653) which conferred to the organ the function to form bile rather than blood. Simultaneously, the first book dedicated exclusively to the anatomy of the liver was published by Glisson (1654) providing descriptions of the vessels and the capsule (Glisson’s capsule) of the liver [1-3]. Since then, important findings have followed one another permitting a better understanding of the liver. Nevertheless, many aspects of liver functions and structure still remain obscure and require accurate investigation, whereas the three-dimensional anatomy of this organ is poorly understood [4].

1.1. MACROANATOMY OF THE LIVER

The liver is a large, second to the skin for extension and weight (measuring about 30 cm through its longest diameter and from 1.80 to 2.30 kg in weight [2]), and structurally and functionally complex organ of the human body, considered second only to brain in its complexity [4].

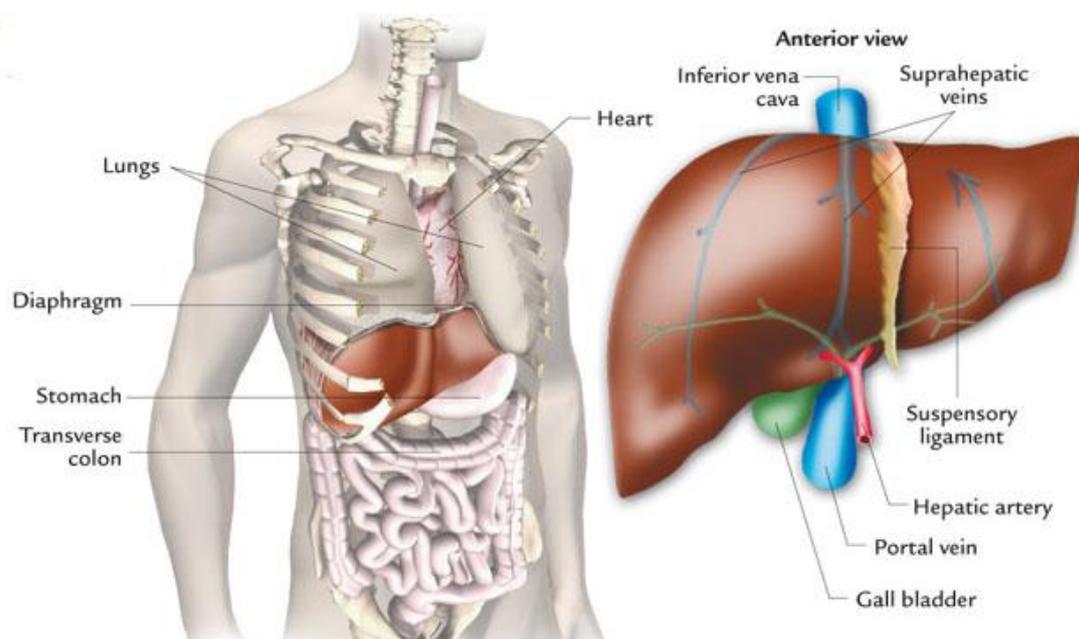


Figure 1. Location of the liver in the body and enlargement of its anterior view. (Image source: <http://knowyourliver.net/location-of-liver-in-body-healthy-liver-and-cancer-2.html>)

The liver is red-brown in color and has a smooth surface due to the presence of a thin connective capsule (Glisson's capsule) [5]. It is located in the upper region of the abdominal cavity and it is bounded above by the diaphragm, anteriorly and laterally by the arch of the ribs, posteriorly by the spinal column, and below by the stomach and intestines. Moreover, a pear-shaped reservoir – the gall bladder – is placed below the liver under surface and receives the produced bile (**Figure 1**) [2]. The liver is held in this position by five ligaments. It is externally divided into five lobes (right, left, quadrangular, lobe of Spigelius, and caudate) identified by an equal number of fissures (**Figure 2.A**) [2, 5]. Alternatively, liver has been recently fragmented into nine segments based on the vascular and ductal branching patterns of the right and left sides (**Figure 2.B**) [4]. Generally, each segment has its own blood supply and biliary drainage. This segmental pattern is especially useful to facilitate surgical resection [6].

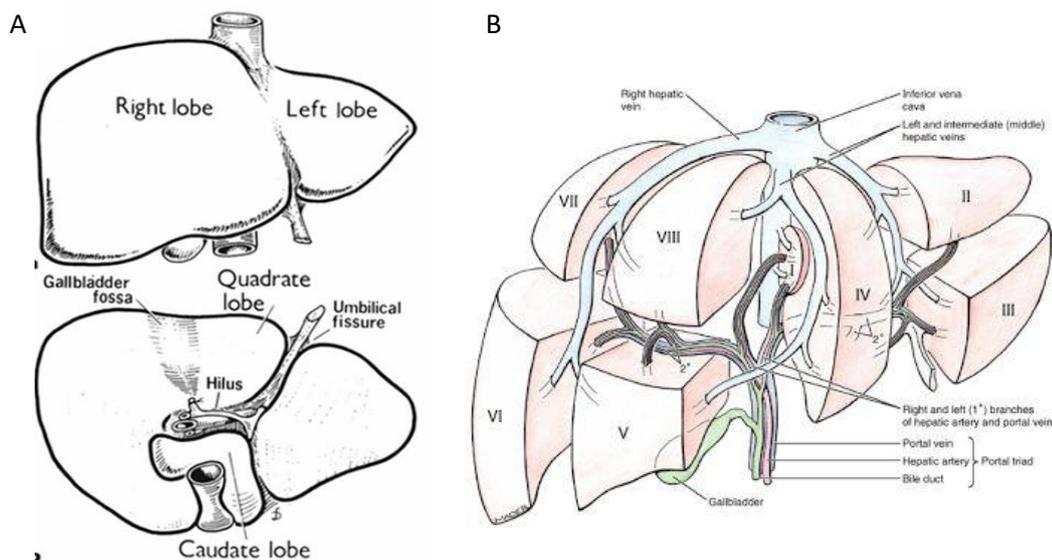


Figure 2. The liver can be schematically fractioned according to different criteria. **(A)** Morphologically, the liver is divided into a right and left lobe (front view), whereas two additional lobes can be identified from underneath, namely the quadrangle and caudate [5]. **(B)** Based on another segmental pattern, especially used in surgical resection, the organ is fractioned into nine segments. (Image source: <http://teachinganatomy.blogspot.com/>)

The liver receives approximately 25% of the cardiac output via two main distinct vascular systems: the portal vein and the hepatic artery. These vessels enter the liver at its hilus together with the hepatic bile duct, lymphatics and nerves. The portal vein is a valveless afferent vessel which carries blood from the spleen, pancreas and intestines. It contributes about 75% of the blood volume to the liver; this blood is rich in nutrients and other absorbed molecules but relatively poor in oxygen content (40%). The portal venous system has been considered as composed by conducting and distributing systems. The conducting one is responsible for getting blood to the farthest corner of the liver. For this purpose, this system possesses frequent branches in the perihilar and subcapsular zones and shows versatility in branch length, number of branching orders, and number of branches. The distributing system is responsible for the exchange of substances between blood and hepatic elements; therefore, it follows a strict pattern of branching. The hepatic artery, a branch of coeliac trunk from the abdominal aorta, contributes about 25% of the blood volume entering the liver; this blood is well-oxygenated (60%) but nutrients poor. Questions regarding its role in perfusion of the liver parenchyma are still open. The dual blood supply of the liver is an

unique feature of the hepatic vasculature and determines the regulation and distribution of blood flow [4, 6, 7]. Blood from the portal vein and hepatic artery mixes as soon as it enters the liver and continues its flow through an interconnected network of specific hepatic capillaries, so-called sinusoids, towards a central vein. While passing through the sinusoids, an exchange of materials takes place between liver cells and blood. The blood, finally collected in the central vein, exits the liver and returns to the general body circulation by means of the hepatic veins. These latter (right, intermediate and left), which also lack valves, and other small hepatic veins drain into the hepatic segment of inferior vena cava. The caudate lobe drains directly into the inferior vena cava [6]. Eventually, blood moves towards the heart by flowing through the inferior vena cava.

1.2. MICROANATOMY OF THE LIVER

1.2.1. GENERAL ASPECTS: FUNCTIONAL UNIT AND HEPATIC SINUSOIDS

For the study of the microanatomy of the liver, a functional unit required to be designated. By definition, functional unit of an organ is the smallest, structurally distinct, “self-sufficient” unit that can independently perform all know functions of that organ [7]. Because of the functional complexity of the liver and its sophisticated three-dimensional architecture, determination of liver functional unit has continued to mystify morphologists for over 300 years [4]. Nevertheless, several models of liver functional unit have been proposed. Those described by Kiernan [8] and Rappaport [9] are perhaps the most well-known and used among all the others.

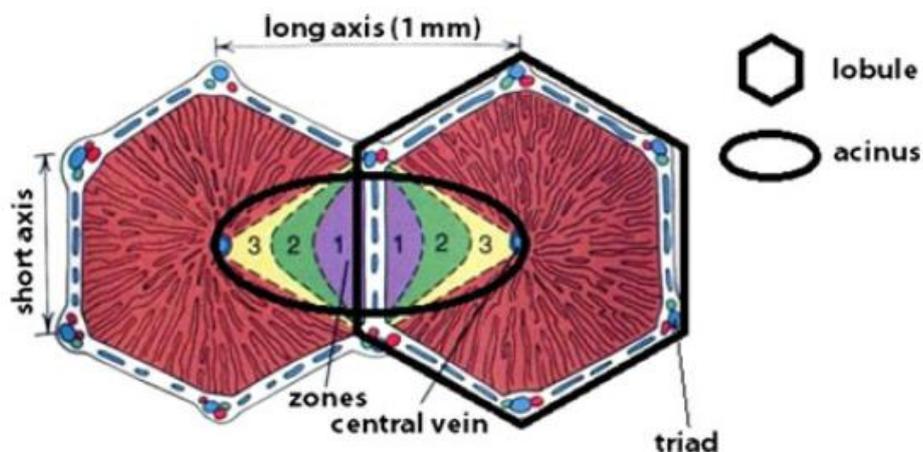


Figure 3. Organization of the liver lobule and acinus. Liver lobule is hexagonal in shape; it presents a central vein in the middle and portal triads at its corners. Liver acinus is oval in shape. Based on the local blood composition, it can be divided into three zones: periportal (or 1), transitional (or 2) and perivenous (or 3). Image source [11].

Kiernan defined the so-called *classical liver lobule* which is characterized by rough hexagonal morphology when observed from a cross-sectional view (**Figure 3**). In the center of the lobule lies the central (or terminal hepatic) vein which collects blood from the lobule and carries it to the hepatic veins. Interlobular branches of the portal vein and the hepatic artery are located at the lobule periphery and supply incoming blood. The bile duct is also present here. Collectively, the portal vein, hepatic artery and the bile duct together comprise the portal triad. About six portal triads are located at the corners of the hexagonal prism and surround the central vein. The classical liver lobules cannot be easily recognized in man and rodents since a scant amount of connective tissue septa extends between portal triads [10].

In the classical liver lobule, blood flows from the portal triads to the central vein. Bile flows in the opposite direction from the central position to the portal triads at the lobule periphery. The classic liver lobule model emphasizes the anatomic structure of the lobule. About one century later than Kiernan's model description, Rappaport proposed the *liver acinus* as the functional liver unit (**Figure 3**). The structure of the acinus overlaps two adjacent classical liver lobules and involves approximately two portal triads and two central veins. An acinus is identified by two portal triads which, if connected by an imaginary line, define the short axis. Two close central veins on either sides of the short axis complete the acinus structure; these make the long axis. Then, the resulting shape is a diamond or oval. Blood still flows from the portal triad to the central vein. But, with that shape of the acinus, the blood flows from the center to the periphery whereas the bile goes in the opposite direction, from the periphery to the center. The liver acinus represents both functional and structural unit which allows understanding liver physiology and pathology [4]. Furthermore, it is important because it emphasizes the metabolic differences in hepatocytes between the portal triads and the central vein. In this context, the acinus can be divided into three zones along its length; these can be discerned following the bloodstream. Periportal zone (or zone 1) lies along the short axis between the portal triads and is perfused with blood rich in oxygen, substrates and hormones. Therefore, cells located here are the first to receive everything in blood including oxygen, absorbed nutrients and absorbed xenobiotics. Perivenous zone (or zone 3) is placed close to the central vein, then, cells are the last to receive incoming blood which is depleted in oxygen, nutrients and xenobiotics. Moreover, a transitional zone (or zone 2) had been included afterwards. It shows intermediate characteristics between the other two zones. However, the model of liver acinus cannot be correctly applied to the human or rat liver because some their enzymatic activities are distributed in a way that contradicts the acinus concept [10].

The liver tissue can be thought as formed by a compact mass of a multitude of lobules (about one million in human liver [11]). At the microscopic level, each lobule is constituted of so-called muralium made of one hepatic cell thick plates, which spread from the portal triad to the central vein, and transmural spaces, that contain tortuous *hepatic sinusoids* (**Figure 4**). Hepatic sinusoids are unique form of capillaries (with diameter of 5.9 μm in the portal region and 7.1 μm in the centrolobular area [12]) which are separated from hepatocytes (detailed description is presented below) by a perisinusoidal space (or space of Disse). Narrow and tortuous sinusoids are found in the periportal area, whereas the sinusoids in the centrolobular region are wider and are arranged in a more parallel way [13].

Beyond the hepatic sinusoids, additional structures can be identified on the hepatic plate. On the side of the hepatocytes opposite to that exhibited to the sinusoids, adjoining hepatocytes weld their faces together through junctional complexes and longitudinal invaginations of their plasma membranes build up *bile canaliculi* (**Figure 4**). Basically, these latter represent dilated intercellular spaces between hepatocytes rather than actual ducts. Hepatocytes secrete bile into the canaliculi. Bile, which comprises the exocrine product of the liver, streams in the canaliculi in parallel to the blood - flowing in the sinusoids - but in the opposite direction towards the bile duct, constituting the portal triad. Then, bile leaves the lobule and is conveyed to the gall bladder by branches of the bile duct where it is stored. During digestion, bile is released from the gall bladder into the duodenum by means of the cystic duct to aid in the digestion of fats.

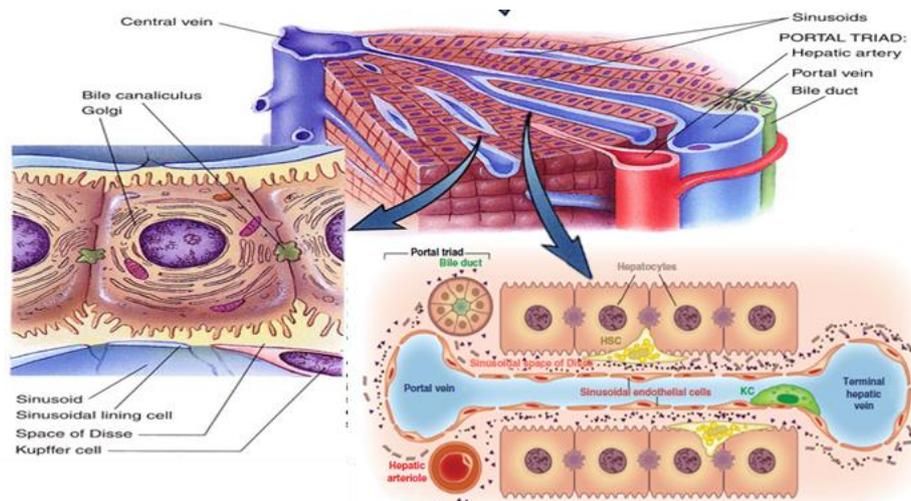


Figure 4. Sketch of a portion of liver lobule and some detailed views of bile canaliculi and sinusoids. Hepatic sinusoids are unique capillaries of the liver. They are constituted by liver sinusoidal endothelial cells and Kupffer cells (KC). The space of Disse separates the sinusoids from the hepatocytes and contains hepatic stellate cells (HSC). (Image sources: <http://tissupath.com.au/education-medical-student-liver/> and [14]).

1.2.2. EXTRACELLULAR MATRIX

In normal conditions, the liver is constituted by a quantitatively very limited extracellular matrix (ECM) and it represents only about 0.5% of the total liver weight [14]. Nevertheless, it has a pivotal role in scaffolding the organ, providing mechanical support, preserving liver homeostasis, maintaining the hepatic cells in a differentiated state, modeling diffusion and vascular flow, and modulating tissue repair. Besides Glisson's capsule, ECM is confined to portal tracts, sinusoid walls, central veins and it is scarcely present between the hepatocyte plates and the sinusoids (so-called space of Disse) [14-17]. Hepatic ECM is regularly constituted by collagens (type I, III, IV, V, VI, XIV, and XVIII), elastin, structural glycoproteins (laminin, fibronectin, nidogen/entactin, tenascin, osteopontin, and secreted acidic proteins rich in cysteine), proteoglycans (heparin sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate, syndecan, biglycan, and decorin), and the free glycosaminoglycan hyaluronan [14, 18]. Around the portal vessels and the large veins, ECM composition is similar to that of other epithelial organs so that laminin, collagen type IV and perlecan can be detected in these areas. In the space of Disse, ECM has a unique spatial expression pattern. Type IV collagen is mainly placed between liver sinusoidal endothelial cells and stellate cells while type I and III fibrillary collagens are situated between stellate cells and hepatocytes (see section 1.2.2. to learn about hepatic cells). Fibronectin and laminin are also present in this location [14, 18]. By contrast, characteristic components of basement membrane are absent in the space of Disse [11]. In the liver, cells and ECM proteins establish bidirectional interactions. Most cells both produce matrix and respond phenotypically to the matrix [17].

1.3. CELLULAR COMPONENTS OF THE HEPATIC PARENCHYMA AND SINUSOIDS

At least 15 different cell types can be found in normal liver (**Table 1**) [4, 13]. The cells composing the hepatic parenchyma and sinusoids represent the largest contributors to the cellular component of the liver. They constitute the exclusive subject of this literature review. Hepatocytes compose the parenchyma of the liver and are the major cellular compartment of the organ. Liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs),

hepatic stellate cells (HSCs) and pit cells are collectively identified as the non-parenchymal cells (NPCs) of the tissue.

Walls of the hepatic sinusoids are formed by a continuous but fenestrated lining of LSECs (**Figure 4**). Sinusoids lack a basal lamina underneath the endothelium. Inside their lumen, hepatic macrophages, better referred as KCs, lie over the endothelium and are attached to this with cytoplasmic processes that sometimes pass through the fenestrations extending into the perisinusoidal space (**Figure 4**). Alternatively, KCs can be present as part of the sinusoid lining. Minor populations of resident dendritic cells and intrahepatic lymphocytes, among which the subpopulation of pit cells, are hosted in the sinusoids. The sinusoids remain separated from the hepatocytes because of the presence of the perisinusoidal space of Disse. This latter appears as a continuous three-dimensional labyrinth of intercellular and pericapillary microlacunae, in which hepatocytes are suspended. It contains many cytoplasmic dendritic projections and cell body of HSCs, bountiful microvilli of hepatocytes, nerve ending and ECM [10, 19] (**Figure 4**).

Table 1 Cell types found in normal liver [4].

Hepatocytes
Biliary epithelia
Endothelia
Sinusoids
Blood vessels (arteries and veins)
Lymphatics
Kupffer cells
Hepatic stellate cells (also known as Ito or fat-storing cells)
Lymphocytes (Pit cells)
Progenitor cells
Oval cell—rodent models
Hepatoblasts—humans
Fibroblasts
Smooth muscle cells (blood vessels)
Mesothelia
Nerves (unmyelinated)
Neuroendocrine cells
Hematopoietic cells
Blood (erythrocytes, leukocytes, etc.)

1.3.1. HEPATOCYTES

The discovery of the hepatocytes (HEPs) dates back to 1824 when M. H. Dutrochet identified “cellules vesiculaires agglomérées” in liver tissue. Few years later, F. Kiernan (1833), J. Henle (1836) and J. E. Purkinje (1837) confirmed the hepatocyte existence and extended their description [20].

Nowadays, it is well-known that HEPs are highly differentiated epithelial cells constituting the most numerous population of the liver (60-65% of the total relative cell number but 80% of the organ volume due to their larger size compared to the other cells) [10, 21]. HEPs are quiescent in physiological conditions, but, thanks to their retained replicative potential, they can proliferate in response to a deficit of liver mass (e.g. partial hepatectomy) or to cell killing caused by chemical or viruses before returning to their quiescence state. In such manner, all HEPs are involved in the fascinating process of liver growth [22]. HEPs are the main actors of the liver performing crucial distinctive activities.

1.3.1.1. BIOLOGICAL FEATURES AND HETEROGENEITY

HEPs build up the parenchyma of the organ by means of a series of branching, anastomosing, perforated plates that radiate from the central vein and form a sort of labyrinth between which run the sinusoids [11, 21]. In each plate, which is usually one cell thick (muralia simplex architecture), HEPs lie adjacent to each other and are firmly joined by junctional complexes.

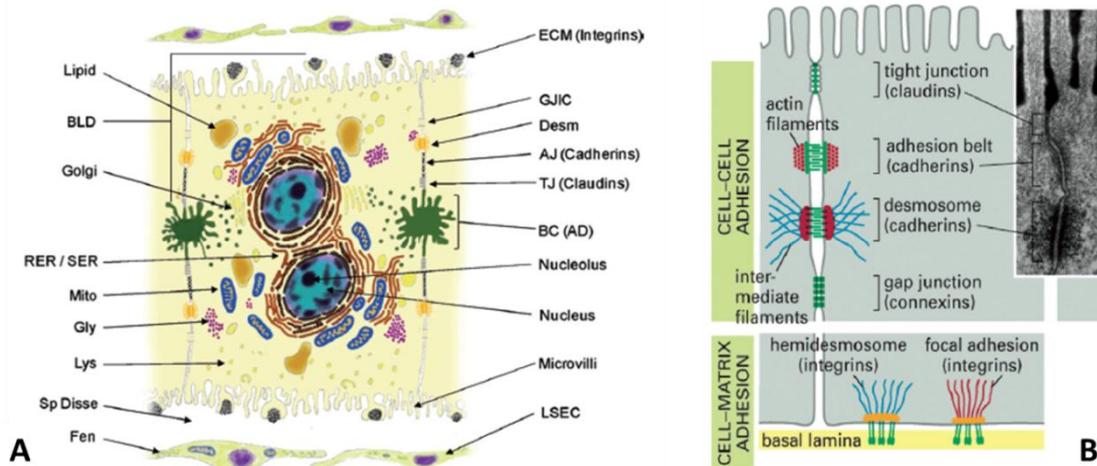


Figure 5. Sketch of hepatocyte. **(A)** Illustration of the morphological features of mature hepatocytes, including rough (RER) and smooth (SER) endoplasmic reticulum, mitochondria (Mito), lysosomes (Lys), Golgi apparatus, and glycogen granules (Gly). Basolateral (BLD) and apical (AD) domains are also indicated. Bile canaliculi (BC) and junctional complexes (gap junction intercellular communication (GJIC), desmosome (Desm), adherence junction (AJ), and tight junctions (TJ)) are presented on the apical domains. Hepatocyte localization with respect to space of Disse and liver sinusoidal endothelial cells (LSEC) is also shown. **(B)** Zoom on junctional complexes established between adjacent hepatocytes, and integrins which allow interactions between hepatocytes and extracellular matrix (ECM). (Images modified from [23]).

HEPs are polygonal in shape (**Figure 5**) and measure about 25-40 μm in diameter [20, 24]. They possess round or oval nuclei, which generally occupy a central position and may contain glycogen in certain conditions (young age and some pathological cases) [24]. HEP nucleus has a double contour with pores that allow interchange with the surrounding cytoplasm [25]. The nuclear content is variable in HEPs. The majority of HEPs are mono or binucleate even though tri and tetranucleate HEPs are occasionally seen in the liver. This increase of the number of chromosome sets per HEP, known as poliploidization, seems to be an age-dependent process; half of HEPs acquires polyploid identity in human adults by contrast HEPs are diploid in young individuals [26]. The fraction of HEPs that are polyploid increases from periportal to perivenous zone [23]. HEP cytoplasm contains huge numbers of a vast array of organelles that is a fundamental aspect of these cells correlated to their high metabolic activity. Specifically, it incorporates over 1000 mitochondria, abundant endoplasmic reticulum (15% of the cell volume), about 300 peroxisomes and 300 lysosomes, approximately 50 Golgi complexes per cell, and an organized cytoskeleton [27]. The mitochondria present a double membrane. The inner one is invaginated forming grooves or cristae where numerous energy-providing processes take place. This is especially due to the presence of enzymes involved in citric acid cycle and β -oxidation of fatty acids. The rough endoplasmic reticulum appears as an apposition of lamellar structures lined by ribosomes. They synthesize proteins, especially albumin, and enzymes. Indeed, the smooth endoplasmic

reticulum, forming tubules and vesicles, contains microsomes and is mainly responsible of bilirubin conjugation and xenobiotics detoxifications. Peroxisomes, which are versatile organelles, exhibit catabolic and biosynthetic roles thanks to their variety of enzymes. Whereas, the lysosomes, membrane-bound bodies adjacent to bile canaliculi, contain hydrolytic enzymes that, if released, could be harmful for the cells. The Golgi apparatus, presenting a set of particles and vesicles lying near the canaliculus, is also considered to be a 'packaging' site before excretion into the bile. The cytoskeleton consists of microtubules, microfilaments and intermediate filaments which, respectively, contribute to subcellular mobility, motility of the canaliculus and flow of the bile, and - last but not least - stability and spatial organization of the HEPs. Intermediate filaments comprise cytokeratins [25]. Another highly specialized component of the HEPs is the plasma membrane that counts a varying content of lipid components, carriers, receptors, and channel proteins. Accordingly, this is characterized by three morphologically and functionally distinct domains which, in turn, define the HEP polarity. Like for other epithelial cells, HEP polarity is well highlighted by defined localization of the cytoplasmic organelles (e.g. the paradoxical location of Golgi apparatus and lysosomes between the nucleus and the apical surface which, in contrast, predominantly serve the sinusoidal pole) [27] and is responsible for the vectorial exchange of macromolecules between two different physiological environments: the hepatic sinusoids and the bile canaliculi [28]. The absorption and secretion of these macromolecules occur at the HEP sides exposed to the space of Disse and indirectly to the blood flowing in the sinusoids, so-called *sinusoidal* (or *basolateral*) *domains*, that represents about 37% of the external area of the HEPs [20]. Multiple irregular microvilli extend from this sinusoidal surface into the space of Disse where they are surrounded by fluid and matrix component; some others, indeed, protrude through the fenestrae of the sinusoids and have direct contact with the blood [20, 27]. By means of microvilli, the available exchange surface is enlarged by a factor of 5-6 [27, 29]. Invaginations with vesicles underneath are also present to this pole [20]. In the sinusoidal domain are localized sodium pumps and (organic ion and drug) transporters, as well as several specific receptors (including those for glycoproteins, immunoglobulin A, and growth factors). Moreover, it represents the site for endocytosis and transmembrane proteins (integrins) which identify matrix components [27]. The bile specialized secretory surface of the HEPs encircle the bile canaliculi and, therefore, is named *canalicular* (or *apical*) *domain*. It is also rich in microvilli and represents approximately 10-15% of the outer HEP membrane [20, 27]. This domain shows adenosine triphosphate (ATP)-dependent export pumps that assure unidirectional and concentrative transport [27]. The remaining external HEP membrane is termed *lateral domain*; it is smooth and contiguous to neighboring HEPs and is primarily but not only specialized for cell attachment and cell-cell communication, hence, it exhibits junctional elements [30]. In fact, tight junctions (zonula occludens) separated the canalicular domain to the lateral one, while, gap junctions (connexins) facilitate direct intercellular communication which are essential to maintain homeostasis, and adhering junctions (intermediate junctions and desmosomes) seal the adjacent adhesion zones of the neighbouring HEPs providing structural support and integrity [20, 23, 30]. The HEP tight junctions are especially important in bile secretion since they function as a bioelectrical barrier with selective permeability for cations [27] and, in addition, keep bile in bile canaliculi avoiding contact or mix with the blood circulation [31].

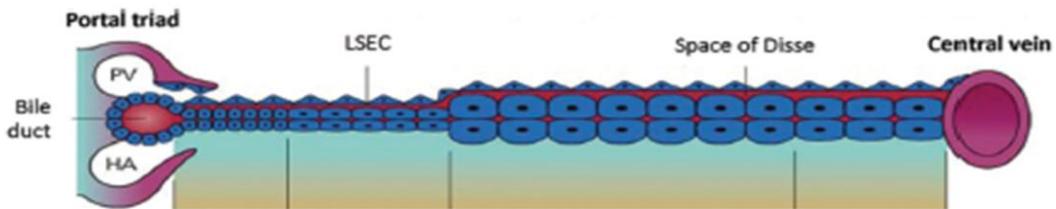
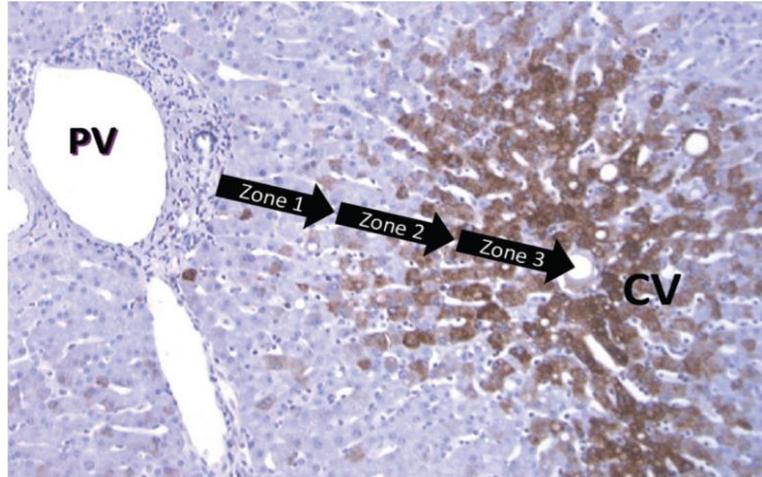
HEPs manifest a remarkable heterogeneity along the portal-central axis of the lobule which results in their different ability to perform functions in different zones of the hepatic lobule [27]. HEP heterogeneity was firstly described by Beale in 1856 who observed a different contribution in secreting bile and in the deposition of 'oil' of various HEPs. Following studies

provided important information concerning the heterogeneous distribution of enzymes in HEPs [32]. Hereafter, several theories were put forward for the purpose of explaining the mechanisms underlying HEP heterogeneity [33, 34]. The most appropriate one ascribed to the blood a fundamental role in this context. Namely, blood entering the sinusoid is a mixture of blood from the hepatic artery rich in oxygen, and portal vein, well-supplied in nutrients. Whereas, hepatic venules presents a low oxygen tension. This generates an oxygen gradient across the sinusoid, thus, periportal and perivenous HEPs are subject to different signals. Accordingly, periportal or perivenous HEPs exhibit different morphological facets [32] and are equipped with different enzymes, translocators, receptors, and subcellular structures [35]. Periportal HEPs are smaller in size (7-15 μ m) and contain higher volume of mitochondria than perivenous HEPs [32]. While passing through the sinusoids, blood undergoes changes in its composition due to parallel occurring of metabolic activities by the HEPs. In this manner, blood in diverse areas exhibits different concentrations of hormones, drugs, or metabolites [33]. Such situation defined the concept of “metabolic zonation”. It was originally proposed for carbohydrate metabolism demonstrating that opposite metabolic pathways, such as gluconeogenesis and glycolysis, could be simultaneously carried out by HEPs residing in periportal and perivenous regions, respectively [27]. The majority of liver functions seem to be zonal. Zonation patterns can be “stable” or “dynamic” and fall into categories of a “gradient” or a “compartment” type [34]. Most exactly, a stable zonation pattern is defined by HEPs exhibiting enzymes whose activity does not change under different nutritional and hormonal conditions (e.g. glutamine synthetase pattern), contrarily, it is possible to describe a dynamic zonation pattern [34]. Indeed, the description of gradient or compartment type of zonation derives from the expression of enzymes in HEPs located in different zones; namely, enzymes present in each HEP but in various amounts or activities define the gradient type of zonation, whereas, enzymes present only in one zone are assigned to the compartment type [34].

Oxygen tension has been identified as the principal regulator of liver zonation [33, 34, 36, 37]. Clear confusion appears in literature about the mechanisms involved in the establishment of zonation patterns by the oxygen. Some claim that oxygen affects metabolic zonation by modeling gene expression chronically in the different lobule areas [38]. Thus, differential gene expression, which strictly involved transcriptional mechanisms, results in a diverse synthesis of proteins in HEPs occupying distinct areas of the hepatic lobule that allow performance of distinct functions [33, 36, 37].

Others conclude that less is understood to this respect and suggest the development of mathematical modeling approaches in order to deepen the knowledge [34]. In addition to oxygen, hormones (e.g. insulin and glucagon), nervous system (sympathetic and parasympathetic regulatin), circadian rhythm (day times and seasons), and morphogens (e.g. Wnt/ β -catenin signaling) also play an important regulatory role on hepatic metabolic zonation [33, 34, 36, 37]. Non-parenchymal cells may represent further metabolic regulators of HEP zonation, but their role in this context has been poorly investigated [34]. Such involvement may be associated with the zonal heterogeneity of the four principal non-parenchymal cell types across the portal-central axis of the lobule [38]. Some main aspects of non-parenchymal cell heterogeneity are presented in Table 2, while details will be exposed in the following paragraphs.

Table 2. Heterogeneity and zonation of the sinusoidal non-parenchymal cell in the hepatic lobule.



	Periportal (zone 1)	Transitional (zone 2)		Perivenous (zone 3)
	7-15 μm	15-20 μm	30-40 μm	30-40 μm
HEP size	7-15 μm	15-20 μm	30-40 μm	30-40 μm
LSEC	<ul style="list-style-type: none"> Fenestrae larger and less numerous 	<ul style="list-style-type: none"> Fenestrae scattered and without well-developed sieve plates 		<ul style="list-style-type: none"> Fenestrae smaller and more abundant Porosity two-fold higher
KC	<ul style="list-style-type: none"> More numerous (43% of total), especially around portal vein branches Larger in size Higher phagocytic activity Higher lysosomal enzyme activity 	<ul style="list-style-type: none"> 32% of total sinusoidal KCs 		<ul style="list-style-type: none"> 25% of total KCs Higher number of galactosyl receptors More active in cytokine production Higher (tumor) cytotoxic potential
HSC	<ul style="list-style-type: none"> Larger amount of lipid droplets \rightarrow higher store of retinyl esters Higher level of desmin 			<ul style="list-style-type: none"> Slightly more numerous Larger cytoplasmic processes

1.3.1.2. LIVER FUNCTIONS

The liver has an important role in many metabolic and excretion processes. Nutrient-containing blood from the gastrointestinal tract travels first to the liver via the portal vein, where nutrients (carbohydrates, lipids, and vitamins) can be removed and stored until subsequent requests. The liver synthesizes and secretes bile, which provides a route for the excretion of endogenous and exogenous compounds (bilirubin, phospholipid, cholesterol, drugs and toxins). It also plays crucial functions in removing toxic materials from the coming portal blood before their entry into the systemic circulation [39]. HEPs perform the majority of the physiological activities commonly associated with the liver, either alone or in collaboration with the four main non-parenchymal cell types [23, 35]. The following paragraphs provide a short overview of certain important liver functions and the implication of the HEP heterogeneity. Interested reader should revise specific reviews [32, 34, 40-43] for additional and deeper knowledge.

❖ LIVER AS A FACTORY

Carbohydrates and lipids share many common features in their metabolism [32, 44]. In both metabolism types, *de novo* molecules can be synthesized from simple metabolites (in the processes of gluconeogenesis and liponeogenesis). Additionally, single molecules can be merged together in forming bigger ones: glucose-phosphates can be converted to glycogen as well as fatty acyl esters can be esterified to glycerides. Finally, glycogen and glycerides can be degraded by pathways that supply substrates for energy production [44].

CARBOHYDRATE METABOLISM

The liver is the glucostat of the body appointed to maintain blood glucose levels within physiological range [32, 45]. It functions as recipient, store, donator, and creator [45]. Periportal and perivenous HEPs play distinctive roles in this context thanks to their exclusive enzyme content and subcellular structures [19].

After a normal carbohydrate-rich meal (absorptive phase), glucose concentration in the body augment. Thus, it is absorbed by the intestine and a little portion is utilized by the brain and erythrocytes under rest condition. Excess glucose is taken up by skeletal muscle, adipose tissue, and predominantly by the liver. This latter takes up more than 90% of absorbed glucose. Glucose passes across HEP membranes via glucose transporters (GLUT-2, 9, and 10) and, in the cytoplasm, is mainly polymerized to glycogen by the perivenous HEPs and stored as such in all HEPs. In brief, glucose is phosphorylated in glucose-6-phosphate by the action of specific enzymes (hexokinase or glucokinase). Glucose-6-phosphate is transformed in glucose-1-phosphate, then in uridine-diphosphate-glucose to be finally stored as glycogen. Glycogen links up to 50,000 molecules of glucose while keeping them easily accessible for reintegration into metabolism [45]. When glycogen stores are enough refilled, perivenous HEPs carry out glycolysis (i.e. glucose breakdown in pyruvate) coupled to liponeogenesis (i.e. glucose conversion into triglycerides) [35, 46, 47].

Conversely, during the short- (after several hours without food) or long-term (after several days or weeks without food) postabsorptive phase there is a requirement of glucose from different districts of the organism. In the first case, glucose is provided by means of two mechanisms, glycogen degradation and gluconeogenesis, performed by periportal HEPs. Periportal HEPs cleaved their glycogen stores in glucose: glycogen is first reverted into glucose-1-phosphate, this latter in glucose-6-phosphate which is converted in glucose [45]. On the other hand, when glycogen storage is empty, HEPs synthesized *de novo* glucose from

other sources, namely, lactate, amino acids, and glycerol. These latter non-carbohydrate carbon substrates are steadily and continuously taken up by the liver and derive from erythrocytes (lactate), protein stores of the muscles (amino acids), and triglyceride stores in the adipose tissue (glycerol) [19, 47]. Whereas, during long-term postabsorptive phase, hepatic stores of glycogen as well as protein stores of the muscle are very limited. Thus, the glucose need of the body is reduced. Glucose is still partially produced by gluconeogenesis and largely replaced by ketone bodies, mainly produced in the mitochondria of HEPs.

Besides HEP heterogeneity, other factors are involved in the regulation of the mentioned processes. The hormonal effect of insulin and glucagon on glycolysis and gluconeogenesis has been extensively investigated. It resulted that, at normal glucose level, insulin activated glycolysis, but had not influence on gluconeogenesis. To the contrary, glucagon inhibited glycolysis and activated gluconeogenesis [19]. This regulation may be due to the preferential stimulation of enzyme expression in HEPs. Such fact was additionally used in *in vitro* studies in order to induce an enzyme pattern typical of perivenous (by utilizing insulin) or periportal (with glucagon) areas in cultured HEPs [19]. Furthermore, glycolysis and gluconeogenesis are probably also under nervous control [19, 34]. Likely, parasympathetic hepatic nerve activity provided auxiliary signals for glycogen synthesis, while, α -sympathetic hepatic nerve activity affects glycogen breakdown [19].

To summarize, periportal HEPs, which receive oxygen-rich blood, are more aerobic and, therefore, can especially perform oxidative energy metabolism. They possess greater mitochondrial volume and key enzymes of glucose release (glucose-6-phosphate, fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase), thereby, they represent the “glucose-forming” cells of the liver [19, 48, 49]. Inversely, perivenous HEPs are less aerobic, therefore, they mainly carry out exergonic processes [35, 49]. These HEPs possess key enzymes of glycolysis (glucokinase and pyruvate kinase type L) and liponeogenesis (ATP-dependent citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase); thus, they can be identified as the “glucose-utilizing” cells of the liver [19, 48].

LIPID METABOLISM

Lipids are essential components in all living cells, functioning as energy store and playing an important role in all biologic membranes [50]. The liver is responsible to synthesize fatty acids (FAs) from acetyl-CoA subunits (which commonly derive from carbohydrate catabolism during glycolysis) and to process them to triacylglycerols (TAGs) [51, 52]. These latter can be stored, contrary to carbohydrates, in an almost unlimited manner into lipid droplets within the HEPs and, in case of body demand, quickly utilized to provide the energy necessary to produce adenosine-triphosphate (ATP) [50]. Alternatively, TAGs, after their production, can be packed in very-low-density lipoproteins (VLDLs) which are, then, exported into the blood stream [51]. The set of these reactions constitutes a biochemical process known as *de novo* lipogenesis. Liponeogenesis is regulated by two mechanisms that are different in terms of time required for them to effect. One, subject to hormonal and nutritional factors, involves long-term, adaptive changes in enzyme activities and, therefore, takes few hours to come into effect. The other one involves short-term modulation of enzymes depending on substrate supply and, thereby, occurs on a minute to minute basis [50].

Furthermore, the liver can collect various exogenous forms of FAs present in the blood stream under different conditions. In regular cases following the absorption of normal meal, HEPs recognize and take up chylomicron remnants¹ from the blood. Chylomicron remnants

¹ In order to facilitate the expulsion from the intestine into the plasma, dietary lipids are first emulsified in the intestinal tract by the action of bile acids produced by the liver. Hydrolyzed lipids are taken by enterocytes and

are finally processed by intracellular lysosomes. The resulting glycerol, FA, cholesterol, amino acid and phosphate residues are metabolized and recycled into new VLDLs [19, 51]. In the fasting state, HEPs recruit free FAs (FFAs), deriving from lipolysis in adipocytes, from the plasma [51]. FFAs can be converted in triglycerides or oxidized. Even in this context, triglycerides can be used to produce VLDLs that are exported in the blood [53]. While, oxidation of FFAs represents a way of energy production. It can take place inside mitochondria and peroxisomes. Precisely, short-, medium- and long-chain fatty acids are oxidized within mitochondria by means of the β -oxidation process, whereas toxic, very-long-chain FAs are oxidized within peroxisomes. In any case, FFAs are generally subject to a first step of activation by acyl-CoA-synthetase to acyl-CoA in the cytoplasm. This allows FAs to cross organelles membranes. Following to a series of reactions involving the actions of specific enzymes in the specific organelle and implying ATP synthesis, acetyl-CoA is obtained. This latter can be further processed via the tricarboxylic acid cycle or, in case of FA excess, be converted into ketone bodies [51, 53].

According to the specific distribution of the enzymes along the hepatic lobule, the different mechanisms involved in the lipid metabolism occur in distinct zones. Liponeogenesis preferentially takes place in perivenous HEPs [19, 32, 54]; in fact, relating enzymes are two-fold higher in this area. This aspect is also strongly sustained by the perivenous presence of glycolytic enzymes, inferring the transformation of excess of carbohydrates to fatty acids [19, 54]. Similarly, production of VLDLs from TAGs occurs in perivenous HEPs [19]. Also ketogenesis appears to be higher in perivenous zone where the corresponding involved enzyme (3-hydroxybutyrate dehydrogenatse) is predominant. It was, thereby, deduced that β -oxidation provides acetyl-CoA for ketogenesis in perivenous HEPs, but acetyl-CoA for energy supply in periportal HEPs [19, 32]. In fact, β -oxidation of fatty acids seems to be active in all acinar zones [32]. However, the higher volume of periportal HEP mitochondria led to conclude that β -oxidation is higher in periportal zone [19, 32, 45].

Besides regulation due to HEP zonation, hormones (especially insulin) crucially affect the lipid metabolism [19, 45].

BILE ACID METABOLISM

The liver is the only organ able to synthesize bile acids [39]. These latter are amphipathic molecules derived from the catabolism of cholesterol which involves multiple steps individually catalyzed by specific enzymes placed in different compartments of the HEPs (endoplasmic reticulum, cytosol, mitochondria, and peroxisomes). Bile acids are extremely important in facilitating hepatobiliary secretion of endogenous metabolites and xenobiotics and intestine absorption of lipophilic nutrients. [55, 56]. Beyond this classical well-studied function, it has been recently demonstrated that bile acids also are signaling molecules that activate nuclear receptors and cell signaling paths that control the metabolism of glucose, lipids, and energy [56, 57].

The enterohepatic circulation of bile acids represents the journey of these latter starting from the liver, passing through the intestinal tract, and ending back to the liver. In short, HEPs synthesized bile acids from cholesterol through the primary action of the cholesterol 7α -hydroxylase enzyme (CYP7A1) [55, 56] that hydroxylates the cholesterol at the C₇ position of the steroid ring [55]. Against concentration gradients, produced bile acids are pushed to cross the HEP canalicular membrane via the bile salt export pump (BSEP) in order

packed into chylomicrons. These latter can finally be expelled into the bloodstream where they receive apoproteins. Thanks to the apoproteins, chylomicrons can deliver TAGs and FAs to adipocytes and myocytes. The chylomichron remanants are taken up by the hepatocytes.

to be secreted in the bile, contained in the bile duct. Similarly, phospholipids and cholesterol cross the HEP canalicular membrane respectively via the phospholipid flippase MDR2 and the ABCG5/G8 transporter, and join the bile in the bile duct [56]. Although many other compounds are secreted into the bile by the HEPs, bile acids are the most abundant and concentrated constituents [39]. The bile flows to reach the gallbladder. Here, bile acids, phospholipids, and cholesterol form micelles to solubilize cholesterol and to reduce bile acid toxicity. After meal intake, gallbladder releases bile into the small intestine where bile acids facilitate the absorption of dietary lipids and vitamins. At the terminal ileum, most of the bile acids are reabsorbed by the enterocytes through an apical membrane transporter and excreted into the portal circulation via their basolateral membrane transporter. Thus, bile acids are carried inside the HEPs across their basolateral membrane, mainly via the Na⁺-dependent taurocholate transporter (NTCP). Within the HEPs, bile acids are made available to be resecreted into the gallbladder [56].

Metabolism of bile acids is zoned on the basis of different aspects. Heterogeneity shows up at the level of gene transcription which is translated in a high prevalence of CYP7A1 in perivenous HEPs implying that these latter are principally responsible of bile acids production [32, 33]. Nevertheless, perivenous HEPs excrete bile acids in a very slow way. This may be due to the low area of bile canaliculi in perivenous HEPs which, by contrast, is considerably higher in periportal HEP. For this reason, biliary excretion is more efficient in periportal HEPs; similarly, zone 1 is also mainly involved in bile acids uptake [19, 32].

PROTEIN METABOLISM

The liver is an important site for the synthesis of proteins made available to the whole organism. It produces cargo/plasma proteins (including albumin, transferrin, lipoproteins), immune-related proteins (proteins of the complement system, acute-phase proteins), and coagulation factors [45]. Plasma proteins are surely the major product of this organ constituting one third of the total hepatic protein synthesis (100 g/day) [19, 49]. Moreover, these represent the principal interaction means of the liver with the other organs.

Albumin is the most concentrated protein in the plasma (60% of all proteins). It is uniquely synthesized by the HEPs (in average, from 12 to 25 g/day [58]) as their predominant product (15% of the total protein synthesis [19]). According to the final usage, albumin production occurs in two different locations of the HEPs: in polysomes bound to the endoplasmic reticulum, in case of albumin excretion from the HEPs into the hepatic interstitium which is mediated by an energy-dependent process, or in polysomes free in the cytosol, when the protein is kept intracellularly for HEP own uses [58]. Albumin synthesis occurs in all HEPs, with a higher concentration in the periportal area [33]. Zonation differences for albumin appear as effect of particular parameters (maturation, age-dependency, ploidy degree, feeding cycle or fasting) [19, 32].

Albumin production follows exclusive pathways which are unlike those of the other plasma proteins. In normal nutrition and hormonal conditions, it is principally regulated by the oncotic pressure sensed by the osmoreceptors of the HEPs in the interstitial environment. However, caloric and nutritional aspects critically influence albumin synthesis. In fact, in case of short-term caloric deficiency, albumin synthesis drops of 50% within 24 hour due to the disaggregation of free and endoplasmic-bound polysomes which implies a decreased rate of initiation of albumin mRNA translation. Nevertheless, the albumin synthetic system is sustained in the HEPs by the maintaining of a regular content of polysomes and albumin mRNA, which allow a rapid recovery mediated by feeding of amino acids. During brief periods of nutritional deprivation, albumin is produced by amino acids derived from

breakdown of intrahepatic proteins. It infers that amino acids stimulate albumin synthesis. However, decrease in albumin synthesis with fasting is not primarily due to decreased availability of amino acids since supply of glucose alone can determine polysome re-aggregation and consequent albumin synthesis. In addition, albumin synthesis depends on the presence of hormones that beneficially increase its production.

Protein synthesis pattern can be altered in HEPs under certain transitory external attacks. For instance, during inflammatory disorders, HEPs reduce the production of some proteins (including albumin and transferrin) in order to employ the saved amino acids in the synthesis of other acute-phase proteins (among which the C-reactive protein) [59, 60]. The process in its globality is called acute-phase response and is initiated by cytokines which are especially released by Kupffer cells of the liver, which provide an effective first-line innate immune defense of the liver (see paragraph 1.3.3), or by macrophages and monocytes at inflammatory sites [60]. Cytokines operate both as a cascade and as a network in stimulating the production of acute-phase proteins. Interleukin-6 is the main pro-inflammatory cytokine which activate HEP receptors inducing their production of acute-phase proteins. Its action depends on the nature or site of the inflammatory stimulus and may require the presence of additional cytokines [60]. Pro-inflammatory cytokines also push HEPs for synthesizing other proteins (such as heat shock proteins that beneficially supervise damaged cellular molecules) and suppressing the P-450 enzyme system [59]. Despite the role of the acute-phase proteins has not been completely clarified, different scientific sources converge on the hypothesis that these proteins may be involved in opsonization and trapping of micro-organisms and their products, in activating complement, in binding cellular remnants, in neutralizing enzymes, scavenging free haemoglobin and radicals, and in modulating the host's immune response [59].

In the liver, protein degradation occurs in the HEPs by two principal mechanisms: i) the autophagic-lysosomal and ii) the ubiquitin-proteasome-related pathways. In the former, a portion of the cytoplasm is engulfed in vacuoles that fuse with lysosomes where the degradable content is finally digested by specific enzymes. Whereas, in the second path, proteins are first tagged in order to be identifiable for degradation, then degraded by enzymatic linkage with ubiquitin residues [45, 61].

❖ LIVER AS A DETOXIFIER

The liver has been identified as the gateway of the body due to its ability to remove a plethora of toxic waste from the blood before that this could reach the other organs.

AMMONIA METABOLISM

Ammonia is directly or indirectly produced by extrahepatic tissues as result of the catabolism of proteins, amino acids, and nucleic acids. Among the several involved organs, the gut is a principal site of ammonia production that is supported by the action of colonic bacteria in hydrolyzing nitrogenous compounds [19]. Ammonia is present in the body fluids as NH_3 and NH_4^+ which are differently transported through the cell membranes, respectively by diffusion and active transport system. In physiological condition, ammonia counts an amount inferior of 35 $\mu\text{mol/L}$ in the blood. Preservation of such low concentration is extremely important because excesses of ammonia in the blood are toxic and eventually lead to the development of neurotoxicity (e.g. hepatic encephalopathy) [62]. On the other hand, ammonia must not be completely removed because it is precursor of all nonessential amino acids as well as crucial metabolite in the mitochondrial redox system [19]. As first identified by Krebs (1932-

1935) [63, 64], the liver plays a fundamental role in regulating ammonia metabolism of the body by detoxifying it through urea and glutamine synthesis [63, 65].

Urea is the major mechanism of nitrogen detoxification and is uniquely formed in the liver by means of two distinct strategies: from catabolism of amino acids in the liver [19] or from ammonia externally produced reaching the liver via the portal vein [49]. Ureagenesis from amino acids preferentially occurs in periportal HEPs, while that from ammonia especially takes place in perivenous area [49]. Blood-contained ammonia ions reach the liver via the portal vein and they first come in contact with periportal HEPs, which manifest enzymes of the urea cycle and glutaminase, and later with those perivenously situated, expressing glutamine synthetase. About 90% of nitrogen excess enters the urea cycle. Hence, portal HEPs convert with low-affinity (but high-capacity) ammonia in urea in an irreversible manner for its final excretion via the kidneys. Urea cycle occurs thanks to the interplaying action of crucial enzymes, five are of extreme relevance, and proteins which participate to a series of reactions, starting and ending from/to the same amino acid (ornithine). In the urea cycle, HEPs also regulate the systemic pH by means of the hydrogen ions (released during the conversion of NH_4^+ in urea) that neutralize the excess bicarbonate produced by the breakdown of amino acids. Glutaminase is a phosphate-dependent enzyme located in mitochondria of periportal HEPs and implicated in the hepatic degradation of glutamine. Activated at physiological portal ammonia concentration, glutaminase contributes to amplify the ammonia concentration in the mitochondria (so-called 'interorgan feed-forward') in addition to that delivered via the portal vein or derived from hepatic amino acids breakdown. In this manner, it importantly regulates the urea-cycle flux in view of the physiologically low ammonia concentrations. Periportal glutaminase and perivenous glutamine synthetase are active at the same time, resulting in periportal breakdown and perivenous resynthesis of glutamine that is a process known as intercellular glutamine cycle. This latter allows adjusting ammonia flux into either urea or glutamine according to the needs of the acid-base situation [62, 65].

The rest of ammonia ions is degraded in the liver by glutamine synthesis that is a reversible mechanism due to the possible release of ammonia from glutamine by the action of glutaminases (as previously explained) [19]. This pathway involves a cytosolic enzyme, known as glutamine synthetase, present in a scant number of HEPs (6-7%) especially located in proximity of the terminal hepatic venules. These HEPs actually eliminate with high-affinity (but low-capacity) the ammonia that was not processed by the rest of the urea-synthesizing periportal HEPs. Thus, these HEPs were named perivenous scavenger cells because of their fundamental role in preserving the physiological, nontoxic ammonia levels in the hepatic vein. Furthermore, these HEPs, devoid of urea cycle enzymes, are responsible of the highest glutamate uptake (70% of the entire liver). However, some 7 to 25% of the incoming portal blood passes by the urea cycle reactions and is directly used for glutamine synthesis.

HEP plasma membrane transporters are equivalently important in the presented detoxification mechanisms [19, 66]. Transport of amino acids across the HEP membranes is ion-dependent. Four transport systems have been defined: L, A, ASC, and N. The former is the only sodium-independent one. Amino acids transport by means of these carriers shows an overlapping specificity and competition of different amino acids for transport by the same system [19]. Ammonia is taken up from the blood by a so-called Rhbg (SLC42A2) transporter. Indeed, glutamate is almost exclusively taken up by perivenous HEPs through the Glt1 (SLC1A2) transporter [66].

DRUG BIOTRANSFORMATION AND METABOLISM

The liver is the primary organ involved in the metabolism of xenobiotics including chemicals and pharmaceutical agents. The general principle of drug metabolism involves chemical reactions that convert the foreign substances into more water-soluble and less harmful metabolites in order to facilitate their excretion. The involved chemical reactions are catalyzed by specific drug metabolizing enzymes (DMEs). Most of these DMEs are present in HEPs in the liver. Thus, HEPs represent the center of the detoxification process of the body [67].

Drug metabolism is a four-steps process which comprises an initial uptake of the chemical from the sinusoidal blood stream by the HEPs (phase 0), its biotransformation through chemical reactions of hydrolysis, reduction, and oxidation (phase I) followed by conjugation reactions (phase II). The final biotransformed metabolites are transported out of the HEPs and released into bile canaliculi or hepatic sinusoids (phase III). Phase 0 and phase III received attention only lately because of their critical role in determining the cellular levels of xenobiotics [42]. Both these phases implicate the activity of basolateral and apical membrane transporters of the HEPs. According to the nature of the xenobiotic, phase I and phase II can be carried out in alternate temporal succession or phase I can be omitted [67].

During phase I, the unwanted compound is partially biotransformed by means of one of three possible chemical reactions (hydrolysis, reduction, and oxidation) into an intermediate or metabolite exhibiting a more chemically reactive and potentially more toxic nature. Thereby, it requires a rapid subsequent transformation. The involved chemical reactions implicate the action of specific enzymes which are lipid membrane bound proteins mainly placed in the HEP endoplasmic reticulum membrane. Most of phase I reactions are of oxidative nature and are catalyzed by enzymes known as monooxygenases, which introduce oxygen into the chemical structure of the foreign molecule creating bioactivated intermediated (such as reactive oxygen species (ROS), also named free radicals). These latter can be extremely toxic. The most common phase I enzymes are called cytochrome p450 (CYP450). CYP450 enzymes are distributed in several districts of the organism, but the liver is their main site (300 pmol of total CYPs/mg microsomal protein) [68]. CYP450 are responsible for approximately 70% of phase I metabolism of xenobiotics [69] and comprise a wide number of versatile enzymes (57 isoforms identified in humans) able to catalyze oxidation of many different substrates. CYP450 enzymes share common features, but each of them has a specific function [67]. Based on their amino acid sequences, CYP450 have been classified in families, among which three (CYP1, 2, and 3) are principally involved in biotransformation of exogenous and endogenous compounds, and subfamilies (labelled with letters A, B, C, etc.). The CYP450 identification acronym exhibits a final number that is specific of the individual enzyme[68]. Most of the investigated CYP450 enzymes show a zoned expression. They are preferentially expressed by perivenous HEPs. However, few isoforms have been identified to be uniformly distributed along the hepatic lobule or predominate in periportal HEPs [35, 40]. So far, CYP1 – CYP3, CYP1A1, CYP1A2, CYP1B1, CYP2B, CYP2E1 and CYP3A have been identified to be involved in the metabolic activation of xenobiotics; some of them are presented below.

CYP1A subfamily includes two enzymes: CYP1A1 and CYP1A2. The former is essentially an extrahepatic enzyme, whereas, CYP1A2, the most well-characterized and physiologically relevant enzyme of this family, is expressed mainly in the liver [67, 68]. CYP1A2 accounts for 13% of the total CYP content in human liver metabolizing about 4% of drugs on the market [68]. Both show strong conservation among species and are inducible [68].

CYP2E1 attracted attention of most of the researchers. This is likely due to its primary involvement in the metabolism of ethanol and other low molecular weight substances, but also in the hepatotoxicity of acetaminophen (better known as paracetamol) [70]. CYP2E1

converts its substrates into highly reactive metabolites that can cause hepatic necrosis. Its expression and catalytic functions are conserved across species [67, 68, 70].

The enzymes constituting the CYP3A subfamily are the most abundant detoxificant enzymes of the human liver [67]. CYP3A4 metabolizes a broad range of xenobiotics including about 70% of all clinical drugs [67, 71].

Phase II aims to bioinactive parent substrates or metabolites deriving from phase I in order to make them non-toxic, water soluble, and, thus, ready to be pumped out of the HEPs. This purpose is achieved by means of conjugation reactions (glucuronidation, glutathione conjugation, sulfation, methylation, acetylation, and amino acid conjugation) that involve the action of different enzymes responsible for the transfer and attachment of a co-factor to the compound (so called conjugation process). To each distinct phase II pathway is associated a specific co-factor. Three phase II conjugation reactions have been identified as the most relevant in drug metabolism and, therefore, they have been well documented. These processes are sulfation, glucuronidation, and glutathione conjugation. Indeed, other phase II reactions are marginally implicated in metabolic pathways for xenobiotics. Brief overview of the former major phase II reactions is presented below.

Hepatic sulfation is a mechanism that increases hydrophilicity of the compounds so that they can be excreted in bile canaliculi or through HEP basolateral membrane for final renal clearance. To this end, sulfate is attached to these substrates. According to the nature of these latter, conjugation step occurs in the cytosol (for small endobiotics) or in the Golgi network (in case of carbohydrates) of, preferentially, periportal HEPs [35]. The enzymes involved in the sulfate conjugation of the substrates are named sulfotransferase (SULT). The cytosolic SULT enzymes comprise three families: SULT1, SULT2, and brain-specific SULT4 which, in turn, are divided in subfamilies. Among all, SULT1A and SULT2A subfamilies play distinctive roles in detoxification. Respectively, the former, evenly distributed throughout the liver, conjugates hydroxyarylamines, whereas, the latter is primarily responsible for conjugation of bile acids [41, 67].

Glucuronidation is an important detoxification mechanism which complements sulfation [41, 67]. To wit, glucuronidation especially occurs in case of saturation of sulfation enzymes due to high substrate concentrations. Consequently, a specific glucuronidation co-factor (i.e. glucuronic acid) is firstly synthesized in the cytosol and conjugated to many of the same sulfated molecules by means of enzymes called uridine diphosphateglucuronosyl transferases (UGTs). The resulting detoxified metabolites (glucuronides) are eventually ready for biliary or urinary excretion. Glucuronidation takes place exclusively inside microsomal membranes of (mainly) perivenous HEPs. In fact, UGTs are anchored via the C-terminus to the endoplasmic reticulum membrane, with the enzyme facing the inside of the lumen. Two families of UGTs have been defined so far: UGT1 and UGT2, both including subfamilies. Among these, UGT1A1 is particularly important because involved in the detoxification of bilirubin [41, 72]. Indeed, UGT2B family plays a role in glucuronidation of many endobiotics. Glucuronidation activity increases from the periportal to the perivenous region of the hepatic lobule.

Glutathione conjugation is a reaction of particular importance because its involvement in the detoxification of potent electrophiles which, otherwise, could covalently bind intracellular macromolecules [41]; therefore, it serves as defense mechanism against oxidative stress [67]. Glutathione is a tripeptide made of three amino acids (glycine, cysteine, and glutamic acid) present in high intracellular concentrations in the HEPs [41, 67]. Because of this elevated amount of glutathione, its conjugation to substrate may occur spontaneously; however, it is more efficient when catalyzed by specific enzymes recognized as glutathione transferase (GST). GST enzymes can be predominantly found in the cytosol and endoplasmic

reticulum, but also in mitochondria, peroxisomes, and nucleus of the HEPs [67]. They are classified depending on their cellular localization. Distribution of GST across the liver lobule is discussion of contradictory findings [19].

Phase 0 and phase III are involved in the uptake of xenobiotics and excretion of biotransformed metabolites across the HEPs, therefore, are responsible of the good performance of detoxification process. Although, HEP membranes show numerous transport proteins, only few play a major role in this context.

Drug uptake is carried out by transporters situated on the basolateral membranes of the HEPs. It principally occurs thanks to members of the superfamily of organic anion transporting polypeptides (OATP) exhibiting a substrate preference for mostly amphipathic organic compounds (including conjugated and unconjugated bile salts, and bilirubin) [43, 73]. Four major OATP isoforms are expressed at the basolateral membrane of HEPs: OATP1A2, OATP1B1, OATP1B3, and OATP2B1. OATP1A2 is expressed at low levels in the liver and play an important role in transporting antitumor drugs [42]. OATP1B1 and OATP1B3 are electrogenic transporters primarily expressed in the liver whose activity is affected by abnormal variations of local pH [42]. They are homogeneously distributed throughout the liver lobule [73]. OATP2, including OATP2B1, are predominantly expressed in perivenous HEPs [73]. Evidently, other minor transporter families are implicated in drug influx depending on the nature of the compound.

Basolateral excretion is mainly operated by members of the ATP-driven multidrug-related protein (MRP) family. MRP3, MRP4, MRP5, and MRP6 are basolateral transporters primarily implicated in pumping organic anions and bile acids out of the HEPs into sinusoidal blood [41, 67]. MRP3 is the most studied transporters. It is responsible for excretion of conjugated (sulfate or glucuronate) bile acids and of glucuronide metabolites. MRP3 expression is as much higher as the increased level of glucuronides to excrete. On the contrary, its expression is not induced by sulfate conjugates. The role of MRP3 in taking glutathiones out from HEPs remains to be elucidated. MRP4 can transport sulfate conjugates of bile acids and steroids with high affinity, whereas glucuronides with lower affinity. It also excretes glutathione conjugates. MRP5 transports a variety of substrates similar to MRP4. MRP6 is localized in basolateral/lateral surface of HEPs and transports glutathione conjugates and BQ123, a cyclic-pentapeptide endothelin receptor antagonist.

Biliary excretion is a carrier-mediated, energy-dependent process. The bile salt export pump (BSEP) is located at the apical membrane of the HEPs and is responsible of the excretion of unconjugated and glycine- and taurine- conjugated bile acids [41]. Hence, it is believed to be the major mechanism able to generate the osmotic gradient of bile acids that determines the formation of a notable portion of bile flow [42]. However, it does not transport phase II metabolites of drugs [41]. The multidrug resistance-associated protein (MRP2) is an ATP-dependent canalicular transporter which excretes many drugs as well as their glucuronide, sulfate, and glutathione conjugates, but also sulfated and glucuronidated bile acids [41]. MDR1 and MDR3 play roles in the biliary excretion of compounds.

1.3.2. LIVER SINUSOIDAL ENDOTHELIAL CELLS

Liver sinusoidal endothelial cells (LSECs) constitute the sinusoidal wall. LSECs comprise about 20% of the total cells but only 2.8% of the volume of the liver. For a long time, LSECs were considered as precursors of the Kupffer cells. Accordingly, both these cell types were thought to possess different functional expressions of one and the same cell type and that they could pass from one function to another by means of transitional stages [13, 74]. Only in 1972, Eddie Wisse presented for the first time the LSECs as a distinct and singular cell

type. Thanks to electron microscopic observations, Wisse evidenced the presence of open fenestration without diaphragm and basement membrane on LSECs; these aspects were later defined as LSEC peculiar features. He also reported the existence of unusual high amounts of endocytic vesicles in the LSECs suggesting that these latter were involved in uptake of protein from blood passing through the sinusoids [75, 76]. Until about 1990, LSECs were especially studied under a biological point of view for the purpose of better understanding and classifying them. After about 1990, LSECs acquired general popularity; scientists of different research fields showed interest in employing these cells in their studies. Since that moment, controversial and confusing descriptions of LSECs have been proposed in the literature [75]. Nevertheless, certain LSEC aspects are at present well characterized.

1.3.2.1. BIOLOGY AND FUNCTIONS

LSECs are hepatic non-parenchymal cells able to self-replicate in adult liver [77]. They show flat, oblong shape and smooth surface, seldom indented by other cells or their processes, with a diameter of around 6.5 μm , when isolated [78]. They are sessile and streamlined cells lying in the direction of the blood flow while forming the endothelium of the hepatic sinusoids. LSEC possesses rather small body with its perinuclear cytoplasm protruding into the lumen or recessing between two parenchymal cells outside of the sinusoidal cavity [77, 79]. LSECs may influence the sinusoidal bloodstream by enlargement of the bulging perinuclear cytoplasm [74]. The perinuclear cytoplasm is not abundant in LSECs and continues laterally into numerous thin, flat and long cytoplasmic processes. Such cytoplasmic extensions form the continuous but fenestrated lining of the sinusoids [10, 12, 13, 74, 80]. The fenestrae are transcellular open pores of about 100-150 nm in size which are devoid of diaphragm. Fenestrae are either dispersed individually across the endothelial processes or organized in groups of 10-100, so-called sieve plates [81]. Alternatively, they appear as interconnected labyrinthine structure [82]. However, porosity, defined as the ratio of fenestrae to the total exposed LSEC surface, ranges approximately between 2-20% per LSEC [81]. Fenestration patterns vary across the liver lobule: in periportal zone, fenestrae are larger but less numerous per sieve, whereas, in centrilobular region, they are smaller but more abundant per sieve plate. In the transition region, along the sinusoid, fenestrae are scattered and without well-developed sieve plates [79, 83, 84]. Hence, porosity is twice as high in the centrilobular area as in the periportal region, allowing increased exchange of oxygen as the $p\text{O}_2$ drops across the lobule [77, 83]. Fenestration is not unique to LSECs but its presence has been documented in endothelial cells of other organs which require unimpeded transfer of substances between blood and surrounding cells [81, 83]. Though, the combination of open fenestrae and lack of a basement membrane makes exclusive the LSEC phenotype. LSEC phenotype is strongly regulated by the vascular endothelial growth factor (VEGF) which is secreted by either hepatocytes and HSCs. VEGF acts on LSECs via two receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGF is predominantly expressed in the centrilobular regions reflecting hypoxia which is the primary stimulus of VEGF production. VEGF control of LSEC phenotype requires autocrine production of nitric oxide (NO) [81]. It occurs through two pathways: a well-described NO-dependent one and a NO-independent path, which remains to be characterized [83]. Because of the absence of basement membrane underneath the LSECs, fenestrae constitute an open connection between the sinusoidal lumen and the Space of Disse through which transport and exchange of fluid, solutes, and particles take place between the blood and the HEPs or HSCs [79, 80]. Therefore, fenestrae play a filtration (sieving) role permitting diffusive and convective

passage of particles of about the fenestrae size. In this manner, LSECs perform a pivotal role in regulating levels of lipids, cholesterol, and vitamin A between the liver and other organs [12]. Many substances are preferentially taken up in periportal areas of the liver lobule [79]. Plasma and substrates within plasma, plasma proteins including albumin, smaller lipoproteins, colloidal particles and polystyrene microspheres can cross sinusoidal wall. On the contrary, bigger particles such as chylomicrons, which, as described above, are lipoproteins involved in the bulk transport of alimentary lipids, cannot pass through the fenestrae. In order to allow the moving of large chylomicrons (200-250 nm in diameter) into the space of Disse, LSECs first have to metabolize these molecules by the action of lipases, enzymes located on LSEC surface, which reduce chylomicrons size by means of the loss of their triglycerides. The resulting cholesterol rich remnants are finally small enough to pass through the fenestrae. By such sieving function, LSECs protect liver against exposure to large quantities of triglycerides conditioning bile secretion and avoidance of atherosclerosis development [10, 77, 80]. The role of the liver sieve has been demonstrated in various diseases, such as hyperlipoproteinemia, cirrhosis and cancer [76].

Wisse described two mechanisms that explain the driving force behind the exchange of particles and fluids between the sinusoidal lumen and the space of Disse [13, 79]. These are named “forced sieving” and “endothelial massage”; their definition is based on *in vivo* observation of the interaction between blood cells and fenestrated sinusoidal wall. This interaction results from the large size of several blood cell types which overlaps that of periportal and, to a lesser extent, centrolobular sinusoids.

Forced sieving rests on the description of the flow of the red blood cells (RBC) in the sinusoids (**Figure 6**). RBCs slowly move in a single row along the sinusoid separated by small volumes of plasma. They easily deform and adapt to the size and shape of the sinusoid while flowing. Between the aligned RBCs, lipoproteins are present in the fluid compartments and are subject to Brownian motion. But their movements are one-sidedly limited by the RBCs’ presence. This interference is thought to enhance the chances of particles escaping through fenestra into the space of Disse. In fact, when these particles are stuck in zones of established friction force between RBCs and the surface of endothelial cells, they roll between red blood cells and the endothelial cell surface, until they pass into fenestrae and disappear into the space of Disse. Therefore, forced sieving promotes the transport of lipoprotein particles into the space of Disse [10, 13, 80].

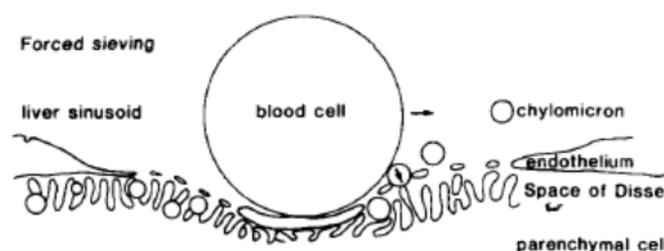


Figure 6 Diagram of the concept of forced sieving of the LSECs [79].

Endothelial massage results mainly from the interaction of white blood cells (WBC) with the endothelial wall (**Figure 7**). WBCs have a nucleus and cytoskeleton, which makes them relatively stiff, less plastic than erythrocytes and unable to adapt to obstacles or diameter changes of the sinusoids. WBCs get stuck into the periportal sinusoids and compress the portion of the endothelial lining, and correspondingly of the space of Disse, on which they are situated. Thus, blood flow is interrupted or retarded. Compression of the space of Disse causes displacement of fluids, which escape through the nearby fenestrae. When WBCs

move away from the occupied sinusoid spot, the endothelial lining and the space of Disse resume their normal shape and fresh fluids are sucked into the space of Disse and start moving in a downstream direction. Thereby, endothelial massage refreshes, mixes, and transports fluids present in the space of Disse [10, 13, 80].

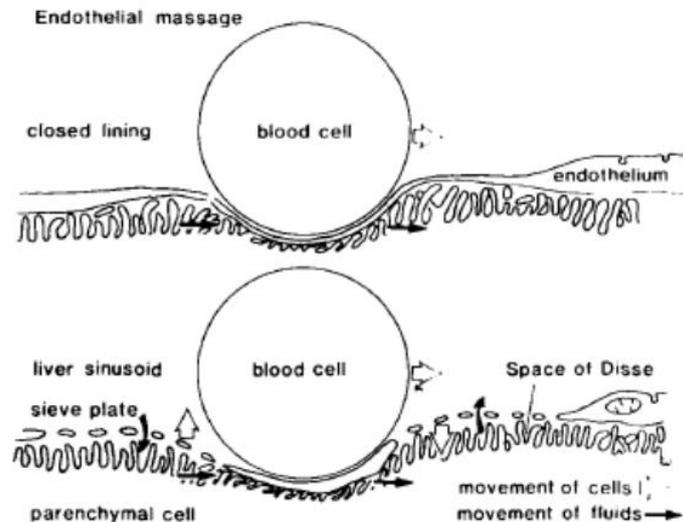


Figure 7. Diagram of the concept of endothelial massage of the LSECs [79].

Both phenomena are more pronounced in periportal zone.

Fenestrae are dynamic structure, whose diameter and number vary in response to a variety of stimuli both *in vivo* and *in vitro* [76]. These changes are of paramount importance in controlling the hepatic functions. For instance, it has extensively investigated the effect of alcohol on fenestrae. It causes increase of diameter but decrease of number of the fenestrae. Chronic ingestion of ethanol and resulting enlargement of fenestrae size may affect lipid metabolism in hepatocytes due to ingestion of larger, triglyceride-enriched chylomicrons and, consequently, the pathogenesis of atherosclerosis [76, 85]. The cytoskeleton plays an essential role in fenestrae dynamics. Braet et al. described the presence of a sieve-associated cytoskeleton and a fenestrae-associated cytoskeleton [86]. The former appeared to be constituted of a circular system, namely a ring of oriented microtubules. Instead, fenestrae seemed to be delineated by a filamentous cytoskeleton perhaps including intermediate filaments, microfilaments or a combination [13, 86]. Such cytoskeletal organization acts as a supporting structure and “muscle” around fenestrae [13]. Its ability to open and close fenestrae in response to specific treatments is linked to the involvement of contractile proteins, i.e. myosin and actin. The presence of actin and myosin filaments in LSECs and their role in the regulation of fenestrae size has been well investigated. Calcium is associated with these proteins, thereby, its intracellular levels play a key role in regulating fenestrations of hepatic sinusoids [13, 81, 86, 87].

It has been shown that drugs which alter the calcium concentration within the LSECs also change the fenestrae diameter, whereas drugs which interfere with the LSEC-cytoskeleton mainly alter the number of fenestrae [76]. Fenestrae are inducible structures. Cytoskeleton and actin are further involved in the *de novo* formation of fenestrae [76, 87].

Usual cell organelles are closely packed together in the LSECs since the scanty cytoplasmic space. Some organelles (Golgi complex, centrioles, and agranular reticulum complex) are confined to the perinuclear cytoplasm, whereas others (mitochondria, pinocytotic vesicles and dense bodies) reside in the cytoplasmic processes. The nucleus - round, ovoid or elongated in shape - is situated along the plasma membrane nearly approaching the space of Disse. The orientation of these organelles, either to the sinusoidal lumen or to the space of

Disse, confers a sort of polarity to the LSECs [74]. Other specialized organelles, such as lysosomes, phagosomes, coated pit, micro- and macropinocytotic vesicles, constitute the pronounced vacuolar apparatus of the LSECs. They are preferentially placed in high concentrations along the plasma membrane facing the blood [10, 74] and attribute a crucial role as a scavenger system to the LSECs [13, 80]. Thanks to these, LSECs transport substances according their size, charge and chemistry. This transfer occurs along two concurrent paths: endocytosis, molecules and delivered to and processed by the endothelial cell self, and transcytosis, particles are transported across the endothelium to the surrounding tissues [76, 88]. By means of their high endocytotic/transcytosis capacity, which represents another singular feature of LSECs [79], these cells free the blood from macromolecular waste products derived from normal turnover processes in different tissues. In this way, LSECs protect the blood circulation from components of the extracellular matrix; for example, they clear sinusoidal blood from collagen that, otherwise, might induce platelet aggregation. There is evidence that the process of endocytosis is different from the mechanism of uptake by the Kupffer cells (see next paragraph). LSECs can take up a wide range of substances after their specific identification ascribed to receptors located on LSEC membrane. Several receptors have been described so far, such as mannose, collagen α chain, Fc, scavenger, and hyaluronan receptors. Hyaluronan, an abundant connective tissue polysaccharide, was the first molecule to be followed during its turnover for the definition of the LSEC scavenger function. It is almost exclusively sequestered by LSECs thanks to a distinct receptor consisting of two polypeptides forming an oligomeric structure. Clearance of hyaluronic acid represents a useful test to verify the correct LSEC functionality. By the hyaluronan/scavenger receptor, LSECs clear hyaluronan, chondroitin sulfate, formaldehyde-treated serum albumin, procollagen type I and III N-terminal peptides, nidogen, acetylated and oxidized low density lipoprotein [83]. Beside specific uptake of certain classes of molecules, LSECs endocytose all kinds of small particles up to the size of 0.1 μm (i.e. colloidal particles). Uptake of larger particles seems to involve carbohydrate receptors [10, 13, 80]. The process of endothelial endocytosis, which determines degradation of the waste molecules, has been summarized in 3 main steps: i) binding and absorption of the ligand over the cell membrane, ii) internalization, and iii) intracellular transport [77]. Briefly, the waste substance (ligand) identifies and binds to its specific receptor or site of the endothelial cell membrane. It has been reported that binding is restricted to bristle-coated pits except for horseradish peroxidase which seems to be able to bind diffusely over the entire luminal membrane of the LSECs. Afterwards, the bristle-coated pit, sequestering the ligand-receptor complex, is pinched off from the cell membrane and internalized into bristle-coated vesicles or micropinocytosis which are located directly below the cell membrane at the periphery of LSECs. Alternatively, phagocytic process is involved for the uptake of large particles. Before the ligand is transported to the lysosomes in the perikarion of the LSECs, it is transferred in perinuclear vesicles showing reduced diameter. During the phase of molecules degradation, a process named retroendocytosis has been identified. According to this, processing of ingested particles remains partial till receptors are not recycled to the LSEC surface. Such recycling provokes dissociation of the ligands from the receptors [13, 77]. The exact nature of the diverse endocytic structures involved in intracellular transportation and degradation of the ligand is still unclear [80].

Besides the above described main functions, LSECs have additional tasks. They seem to contribute at the maintenance of the ECM present in the space of Disse by producing collagenous and noncollagenous matrix components and degrading glycosaminoglycans [77]. The LSECs also play a role in immunity as consequence of the arrival of exogenous antigens in the liver. In the presence of non-harmful antigens, LSECs, thanks to a set of specialized cell

surface molecules (so-called MHC class I and II), function as antigen-presenting cells in order to introduce the antigens to the T lymphocytes and induce liver immune tolerance rather than immune responses [10, 75]. Additionally, LSECs play an active part during inflammation. They control of leucocyte recruitment by producing cytokines, which activate leucocytes and by expressing membrane proteins that allow adhesion of the circulating cells [11, 80]. Another important function of LSECs is to prevent HSC activation and also promote reversion of activated HSCs to a quiescence state, these activities are mediated by paracrine factors [78, 83, 89]. VEGF-stimulated NO production by LSECs supports the preservation of HSC quiescence, but it does not promote reversion of activated HSC to quiescent state [83, 89]. The role of the LSECs in this context has been demonstrated *in vivo* as well as *in vitro* conditions. In fact, replacement of differentiated LSECs by defenestrated LSECs, showing loss of fenestration, does not prevent activation of HSCs. Defenestration of LSECs is a phenomenon caused by multiple different factors and determines i) reduced reactivity of LSECs to VEGF and, therefore, drop of NO production, but also ii) inhibition of retinoid transport to parenchymal cells and HSCs by LSECs. Decreased production of NO stimulates endothelin-1 synthesis by HSCs whereas lack of retinoid uptake from HSCs stimulates their dedifferentiation in myofibroblasts. Thereby, HSCs develop contractility, proliferate and produce more collagen [12, 83, 89]. These episodes result in hepatic fibrosis which is precursor of cirrhosis [76]. Similar effects have been reproduced *in vitro* by adding defenestrated LSECs to culture of HSCs. On the contrary, the presence of LSECs, kept in their differentiated state by VEGF, in culture with HSCs impeded the HSC activation. However, LSEC defenestration can be reversed in both *in vivo* and *in vitro* cases through the removal of the hepatotoxin generating the development of such status and pharmacological intervention (i.e. treatment with a so-called sGC² activator). Return to LSEC differentiated condition is feasible only before the establishment of capillarization, a process associated with defenestration which implies formation of basal membrane bringing sinusoids to resemble to the other capillaries of the body, and the consequent appearance of fibrosis conditions. Mediators implicate in the reversion of activated HSC to quiescence by means of LSECs have not been clearly identified yet [12, 13, 83, 89]. Furthermore, LSECs contribute to liver regeneration after liver injury by inducing *in vivo* hepatocyte proliferation [78, 90].

1.3.2.2. CELL CONTACTS AND CROSS-TALKING

A sinusoidal endothelial cell establishes contacts with other endothelial cells, the microvilli of the parenchymal cell, the HSCs or its processes, the KCs, and the collagen fibrils in the space of Disse. However, these contacts seem to be weaker than those normally established between endothelial cells in other organs. Contact between LSECs takes place at the cytoplasmic processes level, exactly between thin rims, where cell membranes are in close apposition. Any peculiar structure is formed at the contact point but rather LSECs glue one to each other by adhesion molecules. The probable absence of gap junctions is due to the limited contact surface. Thus, recent studies analyzed the interendothelial junctions made by adhesion molecules [91, 92]. Accordingly, both human and rat LSECs seem to express the main transmembrane component of endothelial adherent junctions, i.e. VE-cadherin, *in situ* as well as in isolated cells. VE-cadherin, localized at the intercellular borders, formed functional complexes by association with some catenins supporting the structural integrity, permeability and morphogenesis of the sinusoidal vessel wall. Although typical

² sGC is the acronym of soluble guanylyl cyclase which is the only known receptor for nitric oxide (NO) [Wikipedia source].

transmembrane components of endothelial tight junctions are not present, LSECs also express the zonula occludens proteins ZO-1 and ZO-2.

LSECs touch the hepatocytes through the microvilli arising from these latter cells and present in the space of Disse. On the backside, LSECs are surrounded by HSCs which, with their flat surface or branched cytoplasmic arms, embraced LSECs as pericytes do with capillaries in other organs. Numerous contacts, with sometimes an intervening small piece of basement membrane-like material, are established between these two non-parenchymal cell types; this likely indicate a supporting function of the HSCs for the LSECs [13, 74]. Kupffer cells generally lie upon the endothelial lining far from the perinuclear cytoplasm of the LSECs without establishing specialized junctions but, perhaps, involving adhesion molecules. KCs, which are mobile cells, move along the sinusoid towards other positions where they can insert themselves into the endothelium via pseudopods or larger cytoplasmic processes replacing the LSECs or filling possible interruptions in the lining, through close apposition of the plasma membrane of both cells, in order to preserve the integrity of the sinusoid [13, 74].

Cell communication and cross-talking takes place not only by direct cell contact but also through release and exchange of specific molecules from and between cells. LSECs are able to produce metabolites by which they take part in the local cytokine cross-talk. LSECs supply prostaglandin I₂, prostaglandin E₂, thromboxane A₂, endothelin, Von Willebrand factor, interleukin-1 (IL-1), interleukin-6 (IL-6), and interferon. Prostaglandins and thromboxanes are two different classes of prostanoids which, in turn, are oxygenated derivatives of C₂₀ fatty acids generated by cyclooxygenase pathway. Prostaglandins (E₂ and D₂) have profound effects on hepatocyte metabolism and on hemodynamics. Moreover, prostaglandins of the E type seem to protect hepatocytes from toxic agent [80]. IL-1 α , cytokine primarily entails in immunological and inflammatory responses, promotes adherence of leucocytes in sinusoids and activation of phagocytic function of the endothelium. Instead, IL-6 generates acute phase reaction in cultured hepatocytes [80]. Endothelin-1 (ET-1) and endothelin-3 (ET-3) induce constriction of sinusoids. ET receptors are situated on HSCs. Therefore, sinusoid constriction takes place after ET binding at HSC receptors [13]. Some mechanisms of cross-talking and their effects are not yet clearly explained. For instance, it is not sure if LSECs can activate KCs through secretion of interferon or if they promote acute phase reaction in hepatocytes by secretion of IL-6 [12].

1.3.3. KUPFFER CELLS

Kupffer cells (KCs) are the specific macrophages of the liver constituting approximately 15-20% of the entire cellular population of the hepatic sinusoids but only 2.1% of the total liver volume [93]. They also represent the largest class of tissue macrophages (about 80-90%) in the mammalian body [10].

KCs' first findings occurred in 1876 by means of confusing studies of Carl von Kupffer [94-97]. By a special gold chloride staining method, von Kupffer observed and extensively described star-shaped phagocytic cells (the "Sternzellen") that, later, he identified with the LSECs characterized by endocytotic ability to ingest India ink. The original Sternzellen were actually the cells that are now known as hepatic stellate cells [98]. Only around 1974, Wisse highlighted the von Kupffer's misconception and, by elaborating the previous definition of "reticuloendothelial system" (RES) introduced by Aschoff (1924) and including cells with common origin and capability to ingest colloidal dyes, ended to demonstrate the different identities of LSECs and KCs [94, 99]. However, according to the RES concept, KCs were considered to be endothelial origin cells; this wrong notion remained valid till 1970s when

new histological techniques allowed to unequivocally characterized the four different sinusoidal cells and to understand the KC derivation from monocytes [94, 95].

1.3.3.1. BIOLOGY AND FUNCTIONS

KCs originate from bone marrow monocytes which migrate into the liver and differentiate [98, 100]. KCs reside in the liver often located inside and at the junctions of hepatic sinusoids adhering to the luminal endothelium [94, 101]. KCs are ameboid in shape and motile along sinusoidal wall (4.6 ± 2.6 microns/min) passing in and out of the hepatic space [94, 96, 102, 103]; thanks to this capability, KCs contact circulating lymphocytes and engage antigens [45]. Beyond the presence of Golgi apparatus, coated vesicles, pinocytotic vesicles, ribosomes, centrioles, microfilaments, and microtubules, KCs' cytoplasm possesses dense bodies and vacuoles of various sizes, including lysosomes. Their nuclei are ovoid or indented and occasionally lobulated. KC surface shows microvilli and lamellipodia, rarely fillipodia, which extend their projections in all directions adhering to the endothelium or penetrating the endothelial fenestrae to enter the Space of Disse where they may contact hepatic stellate cells and, seldom, hepatocytes. Other processes extend across the lumen to anchor in the opposite wall of the sinusoid [94, 101]. Because of this spreading configuration, resting KCs appear to be starred in shape. Although KCs communicate with the other hepatic cells, they do not establish any organized junctions. Surface of KCs are also covered with wormlike structures and fuzzy coat that are unique features of these cells. These structures along with the cytoplasmic vesicles, vacuoles, and lysosomes represent fundamental components involved in endocytic mechanisms of the KCs [94].

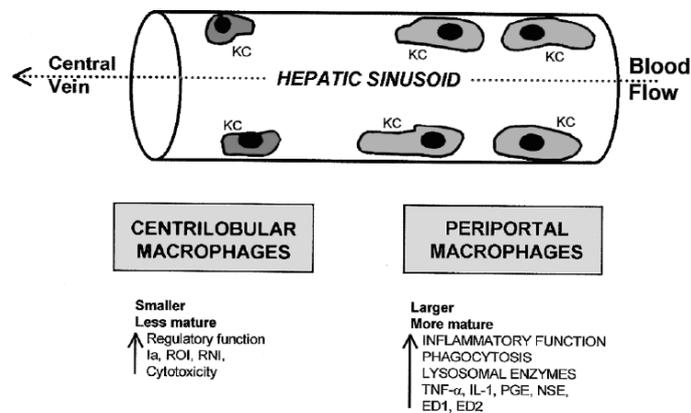


Figure 8. Schematic representation of the heterogeneity of the Kupffer cells [96].

KC size and functionality are heterogeneous and related to their location within liver lobule (**Figure 8**). KCs are about twofold more abundant in periportal (43%) than centrilobular (29%) regions [97]. Periportal KCs are larger, possess greater lysosomal enzyme activities, and are more phagocytic than cells in centrilobular areas but generate less superoxide anion. Therefore, periportal KCs exhibit more scavenging functions and are less active in inflammatory reactions [94]. In addition, this KC fraction produces the highest amount of tumor necrosis factor-alpha (TNF- α), prostaglandin E₂ (PGE₂) and interleukin-1 (IL-1) [100]. Thus, KCs specifically orientate themselves in the liver sinusoids so that their cell membrane faces into the blood flow of the portal vein. Continuously exposed to the portal blood, KCs are in a chronic state of low-level activation [96] and play a major role in filtering, detoxifying, and removing endotoxins and soluble substances deriving from the gastrointestinal tract and accessing to the liver through the portal vein [100, 104]. These

compounds are firstly recognized by KCs thanks to their specific cell-surface receptors [97]. Depending on the size of the ligand, KCs take up the substance by two distinct mechanisms, phagocytosis and pinocytosis. Differently from LSECs, KCs ingest larger particulate matter and insoluble waste by means of by phagocytosis, which is an energy-depend process that involves glucose utilization, increase of phospholipid and protein synthesis, and actin-like cytoplasmic microfilaments engagement in endocytosis after the adsorption of the material to cell membranes [98]. KCs are the primary hepatic cell responsible to engulf particulates larger than 0.3-0.5 μm by their hyaloplasmic pseudopodia [101]. KCs can remove a variety of substances (complement components, immune complexes, cellular and tissue debris, and collagen fragments). Such substances bind to receptors on the surface of the KCs and are rapidly internalized through invaginations of the cell membrane. After ingestion, KCs can swell greatly, possibly causing obstruction of the sinusoid, and may evolve into multinucleate giant cells [93]. In normal cases, the ingested agents undergo further invagination inside the KC cytoplasm and move along tracks of microtubules as long as they are released inside the KCs and modified by both oxygen-dependent and oxygen-independent mechanisms in order to easily transfer them to the HEPs for final disposition. Alternatively, the substances can be directly brought into KC lysosomes for degradation while the corresponding receptors are transported back to the cell surface to be used again or degraded [97]. Binding to specific KC receptors (including Fc, C3, and scavenger ones) triggers additional mechanisms. For instance, scavenger receptors mediate the removal of senescent erythrocytes from the blood circulation [94, 100]. Ingestion of senescent erythrocytes determines the increased expression of the so-called heme oxygenase (HO-1), an enzyme distributed in the endoplasmic reticulum and perinuclear envelope of only KCs in the liver and responsible for the oxidative degradation of heme molecules. The senescent erythrocytes breakdown induces bilirubin production (about 75% of the total amount) and iron release. KCs are, therefore, involved in bilirubin metabolism [94]. Whereas, release of iron at low quantities seems to induce cytoprotective and antiapoptotic effects [100]. In addition, KCs clear the blood from immune complexes thanks to their receptors which recognize the Fc portion of immunoglobulins (IgG, which are also degraded by LSECs, and IgA, which is present in the portal blood flow as major immunoglobulin of the gut mucosa) [97]. HEPs also possess IgA Fc receptors. KCs, as the LSECs, removed many glycoproteins from the circulation. Both cell populations share the same receptors (e.g. mannucole receptor), therefore, they recognize and clear the identical molecules, but with different kinetics. KCs uniquely possess galactose receptors, which only recognizes galactose-terminated oligosaccharides, and fucose receptors, which distinguish fucose-terminated glycoproteins [97]. KCs also contribute to the regulation of cholesterol plasma levels. They have receptors for high- and low-density lipoproteins (the former is also present on LSECs which do not mediate clearance of the corresponding molecules). Low-density lipoprotein receptors are also identifiable on HEPs; however, KCs show the ability to degrade low-density lipoproteins in a measure which is 18-fold higher than that of the HEPs. Through these processes, KCs protect HEPs from attacks deriving from portal external agents in order to avoid subsequent HEP apoptosis. Hence, KCs, together with the LSECs, provide an effective first-line innate immune defense of the liver [45]. Such hepato-protective function is likely mediated by several cytokines and other mediators (such as IL-10 and cyclooxygenase) that are also essential in counteracting inflammatory responses and/or stimulating liver regeneration [105]. The cyto-protective action of the hepatocytes is also supported by KC prostaglandin secretion [98]. PGE₂ attenuates pro-inflammatory signals and enhances anti-inflammatory processes [106]. The KC involvement in hepatocyte protection is especially evident in presence of microorganisms (bacteria and endotoxins) which are

engulfed by absorptive pinocytosis. For example, in presence of the endotoxic lipopolysaccharide (LPS), component of gram-negative bacterial cell walls in the circulation, KCs mediate its uptake in the liver and detoxify it before passing the product to the hepatocytes [97]. However, the clearance of bacteria and endotoxins does not only involved phagocytosis process by KCs, but it is more complicated and depends on complex interactions of KC and neutrophils. Briefly, after bind of the toxic substance to KC's membrane, neutrophils, rapidly immigrated to the infectious site, adhere to the KCs and internalize in order to kill the organism bound to the KC membrane. Neutrophils are subsequently eliminated by KCs in order to resolute the inflammatory condition [100]. The hepato-protective role of the KCs has been observed during drug-induced liver injury and appeared to determine alteration of hepatocyte membrane transport proteins expression. In details, following drug exposure (for example, to acetaminophen, also known as paracetamol), KCs produce inflammatory mediators (TNF α and IL- β) which activate signaling pathways that end to increase the expression of the basolateral efflux transporter (multidrug resistance-associated protein 4, MRP4) while reducing that of uptake transporters (by IL-6 release). In this way, accumulation of toxic chemicals and products of cellular injury may be reduced within the hepatocytes. Alterations in MRP4 may additional promote paracrine signaling to adjacent hepatocytes and other non-parenchymal cells to facilitate the coordinated response of the several cell types. KCs seem also play a role in the basal regulation of organic anion-transporting polypeptides (OATP-1) and multidrug resistance-associated protein 3 (MRP3) [107]. Such defensive role is improved by KC activation.

Hence, KCs principally clear the portal blood from antigens, but also manifest the capability to degrade effete proteins and lipids, to inactivate certain drugs and molecules. By formation of glycoprotein, lymphokines and growth factors, KCs can direct the production of proteins by hepatocytes and their ability of proliferate [98]. In addition, KCs, like other mononuclear phagocytes, can act as antigen-presenting cells for the induction of T-lymphocyte responses [96, 97]. KCs provide important regulatory functions within the liver including development of tolerance to antigens and containment of systemic immune responses, which are extremely important in controlling the inflammation state of the liver [45].

Despite controversial evidence about the activation of KCs [98], these cells seem to undergo activation following direct or indirect bind of several particles and soluble substances (including the complement factor C3a and C5a, or LPS) to their membrane receptors [100]. Activated KCs are subject to gradual different alterations of their phenotype becoming larger and vacuolated. KCs may be activated by bacterial agents or LPS. In this case, activated KCs have enhanced phagocytosis, chemotaxis to standard stimuli and oxidative metabolism; moreover, they are stimulated to release excess tissue-toxic mediators, including cytokines, soluble pro-inflammatory factors, growth factors, and reactive oxygen species which promote influx and activation of neutrophils and may alter the porosity of sinusoids promoting hepatocyte and LSEC damage [103]. By production of harmful soluble mediators, KCs may act as effector cells in destructing hepatocytes and, in this manner, contribute to the pathogenesis of various liver diseases, such as non-alcoholic fatty liver, alcoholic fatty injury, ischemia-perfusion injury, acetaminophen activity, development of liver fibrosis and portal hypertension [100]. For example, release of TGF- β 1 by KCs induces transformation of hepatic stellate cells in myofibroblasts which represents a major step in the initiation of fibrosis [102]. KCs are additionally involved in the stimulation of metalloproteinases and their inhibitors production which is misregulated during fibrosis manifestation causing over-deposition of ECM. KCs also participate in tumor surveillance. It has been demonstrated that

KCs are attracted to tumor cells in the hepatic circulation and are able to phagocytose these cells by reducing the size of the metastases [102].

In *in vitro* culture, activated KCs adhere to and spread more rapidly on culture dishes. [98]. Release of TNF- α can accelerate activation of hepatic stellate cells *in vitro* conditions (see section 1.3.4.1.) [108].

In summary, KCs are hepatic components able to play two major alternative roles in the liver: damage mediators, KCs respond to a toxicant by releasing molecules that mediate the subsequent hepatic damage; and protectors, KCs react in presence of damage by producing cytokines which protect the liver from further injury [103].

1.3.4. HEPATIC STELLATE CELLS

In 1876, Carl von Kupffer identified a population of stellate-shaped cells ('Sternzellen') in the perisinusoidal area of mammalian livers. Initially, he described these as perivascular cells of connective tissue, but, after about 20 years, von Kupffer incorrectly proposed that the cells were a special kind of phagocytic endothelial cells [109]. Afterwards, Zimmerman (1923) observed flattened cells surrounding the hepatic sinusoid with their branching processes and referred to these cells as 'Perizyten' of the liver [110]. Later in 1952, Ito and Nemoto rediscovered these cells and named them Ito cells [10]. Due to the inaccurate observation methods of that time, the two researchers provided an imprecise description of the three-dimensional shapes of the Ito cells assuming that these possessed fusiform bodies sandwiched between the hepatocytes and the sinusoidal endothelium with cytoplasmic processes extended along the sinusoidal wall [110]. Because of the identical determined localization in the liver, 'Perizyten' and Ito cells were supposed to be the same cells. By that moment, several staining techniques were used to characterize these cells and many investigators ascribed different names to them (pericytes, fat-storing cells, interstitial cells, parasinusoidal cells, lipocytes, Ito cells, lipid-storing cells, or vitamin A-storing cells); only in 1996, hepatic stellate cells (HSCs) became the most common and adopted term [109, 111].

1.3.4.1. BIOLOGY AND FUNCTIONS

HSCs are another type of liver non-parenchymal cells which constitute only 1.4% of the total volume of the liver and 6-10% of the number of the hepatic cells [10]; they are present at a ratio of about 3.6 to 6 per 100 hepatocytes [14, 112]. They are distributed almost homogeneously throughout the different zones of the liver lobule, even though some studies describe slight pericentral predominance in normal human liver [32, 111]. Although all HSCs have similar function, they show some heterogeneous features (desmin expression, vitamin A storage, proliferative activity, cytokine and ECM production) [113].

The HSCs are cells of arguable nature. The traditional mesenchymal origin, ascribed to these cells since their morphological features, became questionable when expression of neural and neuroendocrine markers was additionally detected [112, 113].

HSCs are the pericytes of the hepatic sinusoids. They are sessile cells [12] situated under the endothelial lining in the space of Disse, usually associated with collagen-bundles, and, unlike pericytes of usual capillaries, are not enveloped by a basement membrane. HSCs are proximate to the parenchymal cells with their nucleated body, showing oval or elongated nuclei and exactly placed in a recess between two hepatocytes, whilst they are very close to the LSECs with their dendritic cytoplasmic processes that run in the perisinusoidal space parallel to the sinusoidal endothelium [10, 12, 114]. Therefore, these extrusions, which confer a starlike configuration upon HSCs, allow contacts with portion of the liver blood

supply and establish cell-to-cell interactions with other hepatic cells. Two different categories of cytoplasmic processes have been described: intersinusoidal (or interhepatocellular), which are few in number and penetrate the hepatic cell plates reaching the near sinusoid, and perisinusoidal (or subendothelial), which are shorter than the first ones and branch out from the cell body directing along the sinusoid in order to encircle it with fingerlike thorny secondary branches. Besides reinforcing the sinusoidal walls, the perisinusoidal extensions may also sense the surrounding environment detecting chemotactic signals and transmitting them to the mechanical apparatus to generate a contractile force [111]. One HSC can embrace two or more neighboring sinusoids and approach many hepatocytes [10, 109, 113]. HSC cytoplasmic extensions contain a prominent cytoskeleton, oriented along the long axis, and several ultrastructures (mitochondria, vesicles, rough and smooth endoplasmic reticulum, glycogen particles) [10]. The cytoskeleton is constituted of microtubules and intermediate filaments (actin filaments) which are respectively distributed in the core and in the periphery of the HSC's processes and are perhaps responsible for their extension and retraction. Indeed, the rough endoplasmic reticulum and the large Golgi complex, this latter equipped of secretory vesicles, are well-developed as evidence of the HSC active capability to synthesize and secrete proteins [14, 109, 114]. In addition, HSCs produce other connective tissue proteins (desmin, vimentin, and tubulin) and present neural glial fibrillary acidic protein, which is the major intermediate filament in astrocytes [109]. Smooth endoplasmic reticulum is poorly developed, whereas mitochondria are few in number. However, the most characteristic structural feature of the HSCs is the abundant presence of lipid droplets (20.5% of the HSC volume) in the cytoplasm. These lipid droplets are mainly located close to or indented in the nucleus, but also in the cell processes [114]. They especially contain retinol (as retinyl esters, present between 12 and 65% of the total lipid mass), but also tryglycerides (35-50% of the lipid mass), cholesterol (13%), phospholipids (4%), and free fatty acids [115]. Depending on retinoid (vitamin A and its metabolites) dietary intake, lipid droplets can vary in size and number [10, 109]. Two forms of lipid droplets exist into the HSCs: membrane bound, usually smaller than 2 μm in diameter and probably derived from lysosomal bodies; and non-membrane bound, larger than the previous ones (up to 8 μm in diameter) and likely formed from the fusion of multiple membrane bound droplets. Liver is the principal body deposit of retinoids (50-80% of the total amount). HSCs store 80-90% of the total retinol containing in the liver, hence they are the major retinol-storing cells of the body whilst pericytes or fibroblasts in the connective tissues of several organs store very little amounts of retinoids in their large lipid droplets [113]. HSCs accumulate vitamin A in a heterogeneous manner along the hepatic lobule: the store is higher in periportal areas than centrilobular ones [109, 111]. Detection of retinoid droplets can be achieved by exciting them with the light of about 328 nm to which retinoids reply with a blue-green autofluorescence [109, 111, 114]. Retinoids are importantly involved in regulating several cellular activities, such as proliferation, differentiation, morphogenesis, and tumorigenesis, of many cell types [116]. Deficient but also excessive intake of retinoids can provoke development of diseases [117].

Depending on state of the liver, HSCs exhibit *quiescent* or *activated* phenotype. In physiological liver, quiescent HSCs appear as described so far, namely they possess characteristic star-shaped morphology and large lipid droplets storing retinoids. But during liver injury, HSCs transdifferentiate toward a myofibroblast-like cell phenotype. This process is usually referred as 'activation' of HSCs. Activated HSCs lose star shape, lack of lipid droplets, and present new properties, such as α -smooth muscle actin (α -SMA) expression, improved migration and adhesion, increased proliferation, production of chemotactic

substances able to recruiting inflammatory cells, contractility, change in cytoskeletal organization and, most importantly, acquisition of fibrogenic capacity [112].

Quiescent HSCs perform crucial roles in normal liver homeostasis, even though, their exact functions are still controversially discussed [117]. Probably, retinoid storage and ECM homeostasis have been the most investigated functions of the HSCs; nonetheless, other tasks (such as secretion of mediators, contribution to liver development, drug metabolism and detoxification, and liver immunology) are as important as the formers in the hepatic homeostasis. Isolated HSCs, cultured on standard tissue culture plastic, easily lose their quiescent phenotype; this can be avoided by culturing them in suspension on a nonadherent surface [111].

As already noted above, HSCs are the principal depot of retinoids of the body. Dietary retinoids are absorbed by the gut and transported in the form of chylomicron remnant as retinyl esters to parenchymal cells, where are hydrolyzed into free retinol. A small fraction of the retinol may be transformed into different metabolites, whereas the major portion is either rapidly excreted by the hepatocytes then transferred to HSCs or secreted into the blood for distribution to the peripheral tissues. Transferred retinol is reesterified within the HSCs in retinyl ester, thus stored. When body organs request retinoid, the stored retinyl ester is enzymatically hydrolyzed to free retinol and is secreted into the circulation bound to the retinol-binding proteins (RBP). RBP are involved in the exchange of retinol between hepatocytes and HSCs, and viceversa. In summary, hepatocytes are responsible for absorption of chylomicron retinyl ester, metabolism of retinoids, and synthesis/secretion of RBP. Indeed, HSCs primarily carry out the retinoids storage [14, 109, 114].

In addition to the retinoids storage function, HSCs play a crucial role in secreting and remodeling the hepatic ECM in both normal and pathological conditions. Remodeling results from a sequence of matrix deposition and degradation steps both operated by HSCs. In fact, HSCs are the major producers of the ECM components in the space of Disse of normal liver which in turn are responsible for maintaining the HSC quiescent non-proliferative phenotype. Quiescent HSCs secrete laminin and collagen type III and IV, among several other ECM proteins [14]. In addition, HSCs are normally involved in the production of several types of matrix metalloproteinases (MMPs), which are in turn engaged in ECM remodeling under regulation of tissue inhibitors of metalloproteinases (TIMPs). Consequently, HSCs are primarily involved in ECM alterations following occurrence of pathological conditions of liver. In this case, HSCs induce excessive deposition of ECM due to the both excessive production and decreased fiber degradation [112, 113, 116].

HSCs secrete a wide variety of molecules (growth factors, neurotrophins and their receptors, other mediators) used in the interplay with the other hepatic cells. HSCs express and produce hepatic growth factor (HGF) and vascular endothelial growth factor (VEGF) which are potent mitogens of respectively hepatocytes and sinusoidal and vascular endothelial cells. Signaling paths established between HSCs and the other hepatic cells through these secreted molecules may implicitly demonstrated the crucial role of HSCs in preserving homeostasis and promoting regeneration of the liver. Other growth factors are synthesized by HSCs (insulin-like growth factors, transforming growth factor α , epidermal growth factor, stem cell factor, and fibroblast growth factors) which contribute to liver development and regeneration. Among all different secreted mediators, it has been recently emphasized the importance of HSCs in producing immunoregulatory molecules defining the involvement of HSCs in the immune tolerance of the liver [14]. More recently, the involvement of HSCs in regulating liver immunity has been studied. HSCs are a source of soluble immunological active factors, may act as an antigen presenting cell (APC), and have autophagy activity. They also respond to immunological signals and can induce several immune-suppressive

responses. Such implication is associated with the expression of a pattern of recognition receptors by the HSCs [117].

HSCs may participate to xenobiotic detoxification and oxidant stress response of the liver. Such assumption is linked to the expression of alcohol- and acetaldehyde-dehydrogenases³ as well as of some cytochrome P-450 isoforms (CYP2C11, 3A2, 2D1, and CYP3A) by HSCs [111]. Their role in this context is surely minimal compared to that of the hepatocytes, and it seems to be negligible during HSC activation because of downregulation of their cytochrome P-450 [14].

Activation of HSCs is a primary tissue repair response to hepatic injury of various types [115]. This phenomenon roughly comprises two phases: initiation (also known as “preinflammatory stage”) and perpetuation. A third phase, called resolution, may take place if the liver injury completely regresses [108, 112]. The initiation phase consists of those early events occurring in the organization of HSC activation. It arises from paracrine stimulation by neighboring cells (hepatocytes, endothelial cells, platelets, Kupffer cells, and immune cells) which prompts initial fundamental changes in gene expression and phenotype of HSCs. These modifications determine upregulation of membrane receptors making HSCs more susceptible to the different paracrine cytokines and stimuli. Continuous response of the HSCs to these persistent signals eventually promotes the progression to a perpetuation state [14, 108, 112, 118]. Hepatocytes are the main target for most forms of liver injury. Once they are damaged, they release several growth factors, among them transforming growth factors (TGFs), lipid peroxides and apoptotic bodies both contributing to HSC activation. Apoptotic fragments appear to support fibrogenesis of HSCs. In addition, hepatocytes can effect HSC activation by their secretion of insulin like growth factors (IGF) [108]. Vascular and sinusoidal endothelial cells act at the beginning of the process by producing fibronectin and endothelin⁴ 1 (ET-1) as well as activating latent TGF- β ⁵. Fibronectin contributes to the acquired migration behavior of the HSCs, whereas, ET-1 seems to regulate HSC proliferation. Platelets, recognized as potent source of growth factors injured liver, also secrete TGF- β and, in addition, platelet derived growth factor (PDGF) and epidermal growth factor (EGF). T-cells, dendritic cells and Kupffer cells, which strongly interact with HSCs, stimulate HSC activation through secretion of molecules which promote matrix synthesis, cell proliferation, and retinoid release. Among these released substances, reactive oxygen species (ROS) are produced and trigger downstream signals that bring about deposition of collagen. TNF- α , secreted by Kupffer cells, also contribute to HSC activation by enhancing expression of α -SMA and TGF- β receptors as well as promoting synthesis of fibronectin and tenascin. But its effect is not fibrogenic [108]. These described and other changes in the composition and

³ Alcohol- and acetaldehyde-dehydrogenase are enzymes present in some hepatic cells. Alcohol dehydrogenase oxidizes ethanol into acetaldehyde, this latter is subsequently converted into the harmless acetic acid by acetaldehyde dehydrogenase (Wikipedia source).

⁴ Endothelins are a group of isopeptides involved in different metabolic processes, but primarily in vasoconstriction. In injured liver, endothelin-1 (ET-1) is highly synthesized by LSECs and may provide the major contractile stimulus to HSCs. HSC contraction is a predominant cause of portal hypertension.

⁵ TGF- β is one of the most potent cytokine involved in the regulation of HSC phenotype. It consists of three isoforms (TGF- β 1, TGF- β 2, and TGF- β 3). It is produced in its latent form; therefore, it requires activation to perform its tasks. In liver, TGF- β is activated in its mature form by dissociation of its inhibitory latency-associated peptide; this process results from integrins, fibrinogen, and urokinase-type plasminogen activator. The activated TGF- β 1, which seems to be predominately secreted by Kupffer cells, regulates remodeling and apoptosis under physiological conditions. Whereas, it upregulates the expression of collagen I, II and IV, fibronectin and laminin in HSCs in pathological cases. Therefore, the activated TGF- β 1 speeds up HSC activation in injured liver.

stiffness of ECM, provoked by the HSCs and also other cells, further influence HSC activation [14, 108].

Perpetuation is a well-coordinated step of the HSC activation that especially involves autocrine as well as paracrine loops [108] and includes several functional responses including proliferation, fibrogenesis, chemotaxis, contractility, matrix degradation, retinoid loss, and cytokine/chemokine expression. Activated HSCs are, in fact, able to produce lots of soluble mediators (autocrine signals) that regulate many important parameters in HSC activation. Perpetuation phase determinates ECM accumulation and ends with the formation of scar tissue. Proliferation results from misregulation of diverse molecules and HSC receptors. Among them, PDGF, which is the single most potent HSC mitogen identified so far, is highly secreted by platelets and resident hepatic macrophages. Its increased availability causes an augmented expression of PDGF receptors in HSCs which strongly activate proliferation signals. Or also IGFs, released by hepatocytes, support HSC proliferation; this is basically due to the expression of IGF receptors on activated HSCs, which are absent in normal conditions [108]. The number of HSCs with profibrogenic phenotype grows greatly. Because of this enlarged presence of HSCs, production of ECM, and especially of collagen type I, is overwhelming. TGF- β 1 is the major driver of the fibrogenesis process but, obviously, other factors, including cytokines, signaling molecules, chemokines, and cellular stressors, contribute to it. Activated HSCs are able to migrate primarily towards chemoattractant cytokines, recognized thanks to their specific receptors, in order to align themselves within regions of injury. HSC chemotaxis consists in a cytoskeletal remodeling with cell spreading at the tip, movement of the cell body towards the stimulus, and retraction of trailing protrusions. HSC chemotaxis migration is influenced by oxidant signals and hypoxia; as noted above, ECM components (for instance, fibronectin and collagen type I) also contribute and facilitate HSC movement. This mechanism eventually determines the formation of fibrotic septae. Activated HSCs exhibit many phenotypical features of smooth muscle cells; their contraction capability is thereby increased. HSC contractility seems to be a main responsible of portal hypertension in patients with end-stage liver disease. Activated HSCs lose their retinoids droplets. It is not clear whether this phenomenon actively influences HSC activation or whether is an effect of HSC activation. Loss of retinoids droplets may results from a prior loss of specific retinoids receptors on HSCs. In addition, such lack of receptors may not repress the anti-proliferative mechanism in activated HSCs that is normally operated in quiescent cells [108]. Therefore, loss of retinoids droplets appears to influence HSC activation and collagen production. This seems to require cellular autophagy. In other words, hydrolysis of retinyl esters releases fatty acids that are metabolized by β -oxidation, producing substrates that provide necessary energies to start HSC activation [14]. As described so far, activated HSCs undergo changes which support drastic transformations of ECM. However, the ECM represents an important regulator of HSC activation. Among all the modification, HSCs produce especially collagen type I that, with its rigid fibrillary structure, substitutes collagen type IV [108]. This change is associated with the process of capillarization of the LSECs. Activated HSCs additionally undergo change in the expression of receptors (integrins) for specific matrix components. They also overexpress tissue inhibitors of metalloproteinases (TIMPs). Therefore, even though matrix metalloproteinases (MMPs) are secreted by HSCs and are initially essential to facilitate HSC migration towards to sites of the injury, TIMPs deactivate MMPs so that ECM does not undergo degradation. Furthermore, activated HSCs also express a series of chemokines by means they can control immune cell function [14, 117]. To wit, these molecules are chemoattractants for neutrophils and monocytes amplify inflammation during liver injury [108, 117].

The pathological condition of fibrosis can reverse if ECM has not yet undergone some critical changes, i.e. significant collagen cross-linking and development of an insoluble and hypocellular matrix which are manifestations of inception of cirrhosis. Since HSCs provide an important contribution to ECM remodeling in liver fibrosis, the phase of resolution depends on the fate of these cells by experiencing apoptosis, senescence, or reversion to their quiescent stage.

Apoptosis of HSCs determines a reduction of the number of activated HSCs and so of TIMP-1. Therefore, ECM can be partially degraded. Apoptosis can be spontaneously initiated in activated HSCs via specific pathway, but other cells can also contribute to it. For instance, hepatocytes can secrete nerve growth factor (NGF) which is apoptotic towards HSCs. Kupffer cells can promote HSC apoptosis by activation of caspase-9-mediated mechanism. Alternatively, senescence, which seems to be mediated by p53 protein, may lead HSCs to adopt a more inflammatory and less fibrogenic phenotype. At last, reversion to quiescent state generally involves about 50% of the HSCs but entails the establishment of a cell population with high susceptibility to reactivate.

1.3.5. PIT CELLS

In 1976, Wisse et al. [119] provided the first description of the pit cells attributing them such name because of the presence of characteristic granules in their cytoplasm that remind the pits in a grape [27, 120]. The pit cells reside in the hepatic sinusoids and, therefore, belong to the series of non-parenchymal sinusoidal cells. Only in 1983, Kaneda et al. [121] concluded that pit cells were the neutral-killer cells of the liver after noticing their morphological similarities with the large granular lymphocytes [27, 120]. Since then, the number of studies about pit cells raised leading to the attribution of several names to these cells according to different aspects.

1.3.5.1. BIOLOGY AND FUNCTIONS

Pit cells are non-parenchymal sinusoidal cells of the liver counting <2% of the total cellular component of the organ. They are considered to originate from blood neutral killer cells, which are attracted in the liver and pushed for differentiating in pit cells. The pit cells are equally distributed in all lobules of the liver, but heterogeneously in each lobule being predominant in the portal zone [27]. In details, they are situated in the lumen of the hepatic sinusoids where adhere to the LSECs and face directly the blood. In the sinusoids, pit cells are less frequent than LSECs and KCs (one pit cell is identified for every 10 KCs) [120]. The pit cells are not commonly present in the space of Disse, even though they can penetrate the LSEC fenestrae and enter in it with their peculiar pseudopodia establishing contacts with the microvilli of the HEPs [120, 122]. The pit cells also contact KCs and irrelevantly the HSCs [27, 120]. The pit cells are larger in size than other lymphocytes, but share morphological similarities with the neutral killer cells. However, their morphology is very typical. They present hyaloplasm and pseudopodia, indicating that the pit cells are motile cells able to move along the sinusoidal wall [120, 122]. In this regard, pit cells also exhibit chemotactic attraction and high deformability [120]. The pit cells show evident polarity. In fact, their high density nucleus, intended or kidney-shaped, occupies an eccentric position in the abundant cytoplasm with organelles especially located at its one side [27]. Beyond the traditional cell organelles, pit cells present unique structures such as the granules and the rod-cored vesicles. The former are the most perspicuous organelles of the pit cells showing an average diameter of 0.3µm and specific traits. They are spherical in shape, azurophilic, membrane-bound and store lysosomal enzymes and cytotoxic proteins [122]. The rod-cored vesicles are

small inclusions (0.17-0.2 μm in diameter) that are only found in pit cells, although not in all of them [122]. They are placed around the Golgi apparatus and contain a straight rod structure, that bridges the entire diameter of the vesicles, and, likely, cytotoxic factors [27, 120].

Pit cells, as well as the other neutral killer cells of the body, are able to kill certain tumor cells and virus-infected cells without prior sensibilization [27]. They can kill some carcinoma cells which are resistant to the cytotoxic action of activated macrophages and KCs [122]. Furthermore, once activated, pit cells can produce cytokines which allow them to remove pathogens, toxins, and food antigens from the portal blood [123]. Therefore, thanks to their strategic position in the hepatic sinusoids, pit cells may represent a first line of immune defense of the liver and the body [27, 122, 123].

2. PATHOLOGICAL LIVER: GENERAL SCENARIO AND TREATMENTS

The liver is the main filter of the body and as such can be the first target of harmful attacks which can lead to the pathological state of the organ. Beyond disorders associated with genetic transmission (for instance, Wilson's disease and Gilbert's syndrome), liver diseases can occur as consequence of many presenting risk factors (e.g. diabetes and obesity) but common causes are viral infections, high consumption of alcohol, and drug overdose.

A persistent exposition of the liver to these injurious conditions can prompt to gradual (over months (>6) to years), chronic deterioration of the hepatic parenchyma related to inflammatory cells [124]. In response to various chronic liver insults, hepatic cells ultimately trigger the wound healing mechanism. This latter leads to an exacerbated production of ECM that principally exhibits an altered and unusual fibrillar structure of collagen I and III. This is responsible of the encapsulation of the damage (scar formation). In this manner, liver pathologically manages its tissue repair response. The general process is well-known as fibrosis [18, 125]. However, fibrosis can be reversed to physiologic conditions by removing the underlying cause of liver injury [112]. In case of significant ECM accumulation (up to height-fold than in the normal liver) and lack of its degradation, linkage of vascular structures and, eventually, architectural disruption of the liver appear. The architectural disruption of the liver culminates in hepatic cirrhosis and its complications [18, 112]. Cirrhosis is an irreversible condition. Such abnormal physical organization or destruction of the hepatic parenchyma and the incorrect blood flow through the organ prevent the liver from doing its duties. It results in impaired hepatic functions [124]. In its entirety, the pathological scenario has been described as chronic liver failure (CLF). CLF-patients have to be submitted to targeted treatments in order to save the remaining functional parts of the liver. When the liver is severely compromised, it has to be transplanted. Cirrhosis and chronic liver together represent leading worldwide causes of death. For instance, in 2002, they represent the 12th most common cause of mortality in the United States, affecting more than 9.5 per 100,000 people predominantly of male sex [124].

In contrast to the long-term progression of the hepatic decompensation in chronic liver failed patients, manifestation of sudden critical liver damage can occur in subjects who do not have preexisting liver disease. Similar cases define the acute failure of the liver. The acute liver failure (ALF), initially termed fulminant hepatic failure, is a rare disorder that results from severe liver injury characterized by a rapid onset of encephalopathy (< 8 weeks) from the appearance of symptoms [126-128]. A finer classification of the ALF was proposed by O'Grady et al. in 1993 providing a better prognosis on the development of encephalopathy starting from the manifestation of the first symptoms [126, 128, 129]. Accordingly, patients are designated as hyperacute (0-7 days), acute (8-28 days), and

subacute (29 days to < 26 weeks) [128, 129]. ALF is potentially reversible if diagnosed in time. Despite its low incidence (10 cases per million persons per year in the developed world), high mortality and important healthy costs are associated to ALF. Its rarity also implies a limited analysis of the case impeding the establishment of a specific supportive care [127]. Interestingly, causes of ALF are distributed in a different, geographical manner (**Figure 9**). In the developing world, viral causes (with infection by hepatitis A, B, and E viruses) predominate, whereas, drug-induced liver injury (often from paracetamol) is principally responsible of ALF in USA and much of the western Europe [126, 127]. This results from a poor hygiene and sanitation as well as scarce accessibility to vaccination in the developing world in contrast to the easy availability of non-prescription paracetamol (acetaminophen) in USA and Western Europe. However, other pharmaceuticals or substances (e.g. *amanita phalloides*) than paracetamol can cause AFL but the estimated incidence is lower (one or two cases per million people every year). ALF is often associated to a rapid progressive multiorgan failure; therefore, affected patients require to be urgently cured with effective treatments [126].

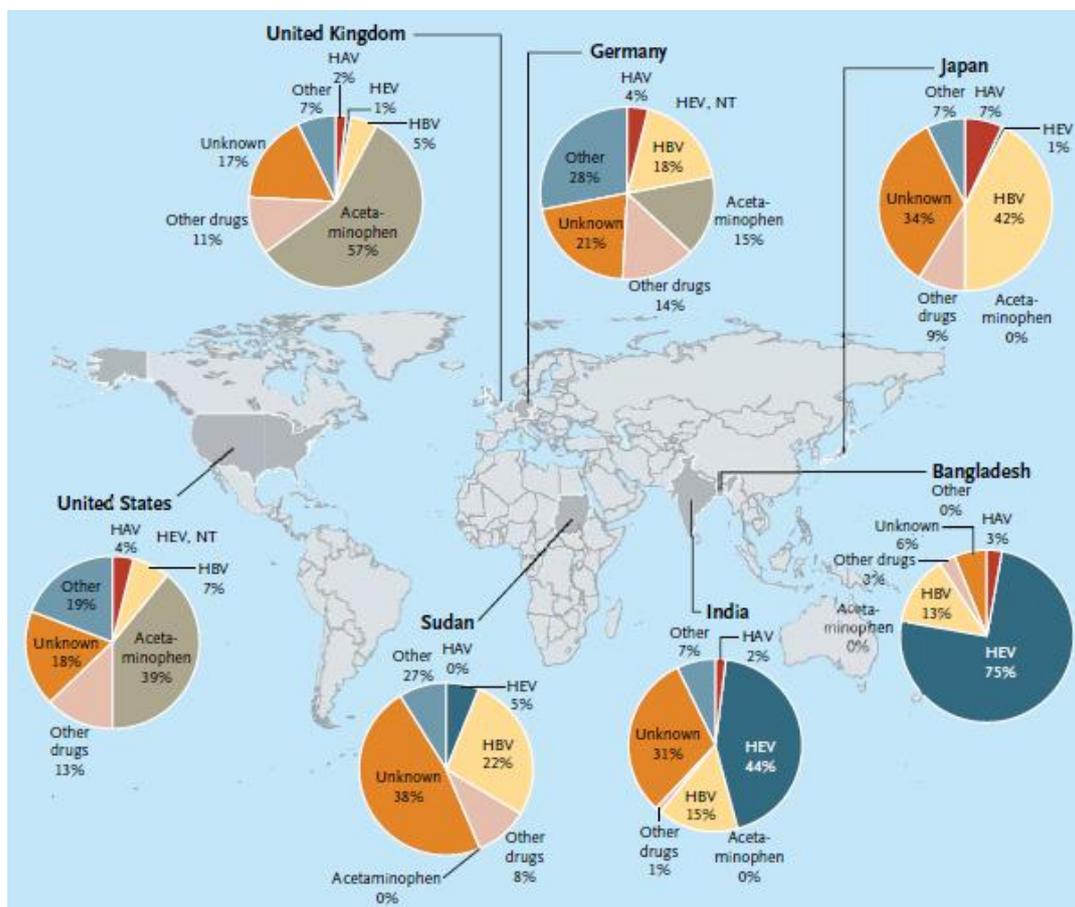


Figure 9. Geographical distribution of ALF causes. (HAV=hepatitis A virus, HBV=hepatitis B virus, HCV=hepatitis C virus, NT= not tested) [127].

A distinct form of liver failure is called acute-on-chronic liver failure (ACLF). ACLF was first defined in 1995 as a condition in which the liver is subject to two simultaneous insults: one chronic and another acute. Although there are neither accepted diagnostic criteria nor based evidences for ACLF, this latter has been referred as acute deterioration of preexisting, chronic liver disease, usually related to a precipitating event and associated with increased mortality at 3 months due to multisystem organ failure [130]. However, the definition of ACLF remains ambiguous and varies among the different research groups. Hence, ACFL

needs to be uniformly described in order to better understand its pathophysiology, prognosis, and treatments [131].

2.1. TRANSPLANTATION AS GOLD STANDARD TREATMENT FOR CLF AND ALF PATIENTS

Orthotopic transplantation is the only current definitive treatment for end-stage livers unable to alternatively recover in both cases of CLF and ALF. Transplanted patients can return to a normal quality and length of life once the liver has recovered by re-acquiring regular structure and functions. Nevertheless, some complications can occur which are often associated to the immunosuppressive therapies and to problems of adjustments of the patient to his “new life” [132]. Unfortunately, transplantation is not universally available due to the shortage of suitable organs in comparison with the enormous request. Therefore, a significant number of patients die while waiting for transplant. Furthermore, in case of ALF, less than 10% of patients are eligible to be transplanted since the rates of survival after the surgical procedure are very low [127, 133]. In fact, orthotopic liver transplantation usually improves from only 1 (in 79% of the cases) to 5 years (in 72%) the survival of the ALF patients. Most of the deaths occur within 3 postoperative months due to infections as consequence of immunosuppression [127].

To overcome the shortfall of donor organs, new techniques have been developed. These comprise the split-liver transplantation and living donor liver transplantation. The former, first proposed at the end of the years 1980 by two different groups [134, 135], consists in splitting a liver graft from a cadaveric adult donor into two parts and, then, transplanting each one in two distinct recipients (usually a pediatric and an adult). Certain data reported equivalent patient survival rates for split-liver transplantation and whole organ transplantation, especially in patients with less urgent status [136]. The second strategy is based on transplanting only a portion of the liver obtained from a healthy donor to a sick recipient. This procedure has been principally used for pediatric patients who received the partial organ from one of the parents, however, some cases of adult recipients are also reported. This technique presents many drawbacks associated with the potential dangers to which the living donors are exposed [136].

To the purpose to find a substitute source of organs to the scarce human livers, the attention was paid to xenogenic livers from nonhuman primates and pigs. Few cases of whole organ xenotransplantation were performed between 1966 and 1973, 1992 and 1993, and more recently in 1995, but these all notably failed [136]. Since then, the use of xenogenic liver tissue in clinical studies has been discouraged by the Food and Drug Administration (FDA). This is also due to the enormous number of risks associated to the xeno-grafts. In fact, in the case of pig livers, which exhibit the advantages to be highly available, cheap, and size matching with the adult human liver, many contraindications have been highlighted, including the elevated probability of rejection of the transplanted organ, the risk of transmission of infectious agents of animal origin to the human recipient (zoonoses), the insusceptibility of porcine hepatocytes to many human diseases, the over production of porcine proteins which have little or no functions in humans, and the possible inability of porcine hepatocytes to detoxify human blood [136]. Nevertheless, researches still go on in order to modify and adapt pig livers for future human transplantation.

2.2. ALTERNATIVE FORMS OF TRANSPLANTATION

Because of the shortage of liver donors, alternative strategies have been proposed at the aim of buying time either to support patient’s liver while waiting for orthotopic transplantation or to enable the recovery of acute failed liver. In this context, two

techniques of auxiliary partial liver transplantation have been developed. A first one is termed heterotopic auxiliary liver transplantation and consists in placing a partial graft below the unresected native liver. Whilst, the another one is called auxiliary partial orthotopic liver transplantation, in which the left or the right hemiliver of the sick liver is resected and substituted by a partial liver graft. In both these procedures, the native organ is preserved allowing it a chance to regenerate, while the auxiliary partial transplanted liver bridges the patient till the healing of his own organ. Once the ill native liver is regenerated, the immunosuppressive therapy is gradually suspended [133] and the donor allograft can be either removed or allowed to atrophy [136]. However, several drawbacks are also correlated to these transplantation techniques, which account from the risks connected to the surgery to the efficiency of the liver regeneration. Although the second approach is technically easier and provides better outcome than the first one, several problems remain unresolved in the clinical application of this procedure (such as the functional competition resulting from the portal blood flow shared between the graft and the native liver) [133].

To the same above mentioned purpose, a different type of transplantation employs only hepatocytes for treatment of ALF. First similar attempts dated back to 1934, when Cameron and Oakeley autotransplanted rabbit liver cells into the peritoneum and under the abdominal skin [137]. At present, this technique consists in isolating hepatocytes from resection of human liver considered unsuitable for orthotopic transplantation and, then, introducing them into the splenic or hepatic portal vascular bed or peritoneal cavity of the patient in order to sustain the hepatic functions in place of the acute failed liver. Many potential advantages are associated to this procedure. Behind the beneficial role of the infused hepatocytes in giving support until the native liver, left in place, recovers, this strategy is surgically easier than the previous described transplantation types and, therefore, entails minor risks for the patient [136]. On the other hand, evident problems are mainly related to the questionable ability of the reduced infused mass of hepatocytes (usually only 5% of the theoretical liver mass) to substitute and support the hepatic functions of the diseased liver. Moreover, the preservation of viability and functions of the these hepatocytes is difficult in such sick environment of the patient [126, 127]. Although this therapy needs further improvements, it may represent a means to sustain hepatic functions in patients waiting for definitive liver transplantation [126].

2.3. OTHER “BRIDGING OPTIONS”

Another category of therapeutic solution is represented by the extracorporeal liver support devices that, similarly to the previous approaches, act by replacing liver functions in patients with ALF in order to bridge them until either a liver donor is available for transplantation or the native organ spontaneously regenerates [126, 132]. Over the past 50 years, many proposals and changes have been done in this field. Nowadays, it is possible to distinguish two distinct big families of extracorporeal liver support devices: artificial (or non-biological) and bioartificial (or hybrid) ones.

2.3.1. ARTIFICIAL DEVICES

Non-biological artificial liver support devices are essentially dialysis-based systems that basically detoxify the blood of the patients from toxins. Therefore, their operation principle is based on filtration or absorption of the toxic molecules through membranes and absorbents. For many years, haemodialysis and haemofiltration, haemoperfusion over charcoal (solely, coated with albumin, or encapsulated in hydrophilic gels) or resins were utilized but their clinical success was irrelevant [132, 136]. An example is provided by the

HemoCleanse Biologic-DT system [138, 139]. Later, a new category of non-biological artificial liver support was introduced. This was brought to light by the acquired evidence that albumin has a high capacity for transport of molecules (including water-insoluble toxins) [140]; thus, albumin was used as a dialysate in these systems [136]. Since then, non-biological artificial liver support devices were specific for albumin bound substances, which represent the majority of the toxins accumulated in ALF patients, and, thereby, more effective regarding their detoxifying potentiality [132]. Two main systems are described below.

In 1993, Stange and Mitzner developed the molecular adsorbed recirculating system (MARS[®], Gambro, Sweden). It uses albumin dialysis to clean out the blood from water-soluble and protein-bound toxins. The blood of the patient passes through a hollow fiber dialysis module (**Figure 10**) where it is dialyzed across an albumin-impregnated polysulfone membrane (cut off limited to kDa to avoid passage of endogenous albumin, hormones and carrier proteins through the membrane). Thus, water-soluble and protein-bound toxins are exchanged by the albumin-coated membrane against 600 mL of dialysate contained 20% of 'recycled' exogenous albumin, which is maintained at a constant counter-current flow in an extra-capillary compartment. Next, the toxins-enriched dialysate passes first through a haemodialysis/haemofiltration module, where it is cleaned from water soluble substances, and then through adsorber columns containing activated charcoal and anion exchange resins, in which albumin-bound toxins are removed. In this way, the dialysate is clean and can be re-used [132, 136, 140].

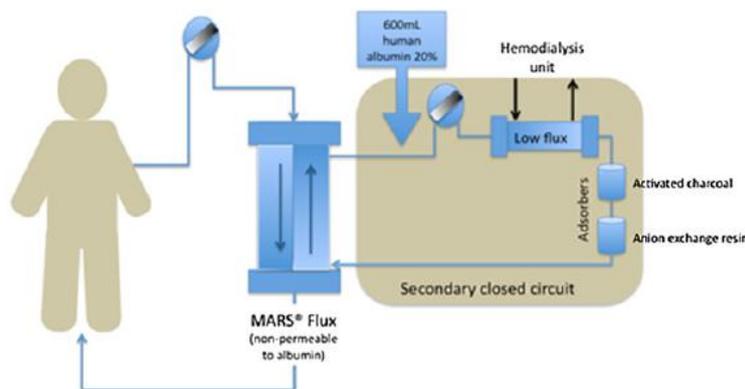


Figure 10. Schematic representation of the MARS[®] device [140].

Several phase I trials of the MARS[®] system have been published so far, involving patients affected by different pathologies including ALF, CLF, and ACLF. Although some clinical trials reported improvements of the liver functions (ammonia, bilirubin) [141, 142] and enhancement of the hepatic encephalopathy [143], no overall benefit of the MARS treatment was demonstrated on the patients survival [136]. However, the MARS treatment revealed to possess a safety profile for the patients [132].

An alternative non-biological artificial liver support device is the Prometheus[®] system (Fresenius, Germany) that was introduced by Falkenhagen et al. in 1999 [144]. As the previous device, Prometheus[®] system is an albumin dialysis and removes water-soluble and protein-bound toxins. Its operation mode is different from that of the MARS device (**Figure 11**), because it works on the principle of fractionated plasma separation and adsorption (FPSA) coupled with high flux haemodialysis [132, 140]. The blood of the patient passes through a first filter (AlbuFlow), which is made of polysulfone hollow-fibers and is permeable

to albumin and albumin-bound substances. Then, the albumin filtrate moves in the FPSA circuit where passes through a first column containing neutral resin (prometh01) and another one having and anion exchanger resin adsorber (prometh02). In this manner, the bound toxins are captured by direct contact with the adsorbing material and the endogenous albumin can return to the patient. The blood is finally dialyzed through a high-flux hemodialyzer with a 250 kDa membrane, where water-soluble toxins are removed [140].

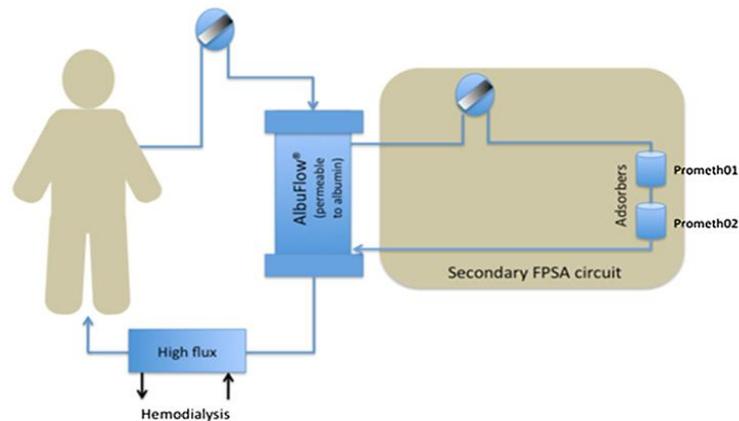


Figure 11. Schematic representation of the Prometheus® device [140].

It has been claimed that Prometheus® system performs a better filtration of albumin-bound substances than MARS® device. In some clinical trials, this device allowed a decrease in the level of some serum components (such as, bilirubin and ammonia) but no improvement in degree of encephalopathy was reported [136]. However, most of these studies involved ACLF patients and only few others ALF ones. As for MARS® system, Prometheus treatment revealed to be safe for the patients [132].

2.3.2. BIOARTIFICIAL DEVICES

The first predecessor of the current bioartificial liver support devices was probably described in a study of Kimoto in 1959 [145]. Nowadays, bioartificial liver support devices (BALs) are generally defined as extracorporeal systems that not only allow the detoxification of the blood of the patient but also perform synthetic and metabolic functions of the liver. To this end, BALs include a biological component, viz. the hepatocytes, which is housed in a “hollow container” so-called bioreactor. The bioreactor is the central feature of the BALs in which the blood or the plasma of liver failure patient circulates and is principally processed by the hepatocytes; these latter are immobilized in it by means of supports (for instance, membranes) preventing efflux of the cells into the patient’s circulation [136]. The treated blood or plasma is finally returned to the patient’s circulation. In addition to the bioreactor, BAL devices employ other components, such as charcoal columns or oxygenators. The ideal bioreactor design would maximize mass transfer to the hepatocytes, so that nutrients and oxygen as well as toxins from the patient’s blood or plasma could efficiently reach the cells [146]. Based on the choice of the mode to anchorage the hepatocytes within the bioreactors, three different categories of bioreactor designs can be distinguished: membrane-based, perfused beds/scaffolds, and entrapment-based [147] (**Figure 12**).

Hollow Fiber	Flat Plate and Monolayer	Perfused Beds/Scaffolds	Encapsulation and Suspension
Pros: attachment surface, potential for immunoisolation, well characterized, cells protected from shear	Pros: uniform cell distribution and microenvironment	Pros: ease of scale-up, promotes 3-dimensional architecture, minimal transport barrier	Pros: ease of scale-up, uniform microenvironment
Cons: nonuniform cell distribution, transport barrier with membranes or gels	Cons: complex scale-up, potential large dead volume, cells exposed to shear, low surface area-to-volume ratio	Cons: nonuniform perfusion, clogging, cells exposed to shear forces	Cons: poor cell stability in suspension, transport barrier due to encapsulation, degradation of microcapsules over time, cells exposed to shear forces

Figure 12. Pros and cons of the bioreactor designs. Image modified by [148].

2.3.2.1. MEMBRANE-BASED BIOREACTORS

Several bioreactor configurations have been proposed so far by different research groups and include hollow-fiber or flat sheet hemodialysers as well as plasmafilters [147]. Some of them, their main features and application in clinical studies, are reviewed below.

The extracorporeal liver assist device (ELAD) consists of a column containing hollow fibers through which the blood flows and (200 g) human hepatocyte cell line (C3A, which is a sub-clone of HepG2 hepatoblastoma cell line) grown in the extra-capillary space. By means of this configuration, a portion of the patient's plasma is ultrafiltrated through the membrane (70 kDa) that also acts as immunological barrier [149, 150]. An oxygenator is placed immediately before the bioreactor. ELAD was used in some clinical trials which demonstrated the safety of the system [150] but no beneficial decrease in plasma ammonia and bilirubin levels [149].

Similarly, the HepatAssist system contains 0.2 μ m-pore hollow fiber membranes, whereas, employs (5-7 x 10⁹) cryopreserved porcine hepatocytes which are attached to microcarriers. In this system, the bioreactor hollow fibers are perfused by the patient's plasma. Therefore, before reaching the bioreactor, plasma is first separated by continuous centrifugation and then passed through an activated charcoal column [149, 150]. An oxygenator is placed immediately before the bioreactor. Large controlled clinical trials were conducted by using the HepatAssist system. First of all, the system appeared to be safe. Other main outcomes are shortly summarized: no porcine viral transmission was reported in the patients after the treatment, certain blood molecules (such as ammonia and bilirubin) decreased in level, neurological improvements were observed in the patients, an important number of patients were bridges to transplantation [132, 149]. However, it was claimed that this device presents some weak points concerning the hepatic function performance: i) reduced amount of used hepatocytes (only 5% of the total liver mass); ii) short duration of the treatment (6-8 hours); iii) pure diffusion involved in the mass transfer between the cells and the plasma [149].

The liver support system (LSS) is formed by a unique bioreactor constituted of four capillary membranes, which are woven into a polyurethane housing. Each of these capillary is designed to carry oxygen, nutrients, or plasma (one for the inflow, the other for the outflow) to the hepatocytes. Thereby, in contrast with the previous systems, the oxygenator is integrated in the bioreactor. Indeed, the hepatocytes (porcine or human, this latter obtained from discarded donor livers) are located externally to the capillaries and spontaneously aggregated [149, 150]. For a phase I clinical study, the LSS was combined with a MARS. The

final device, so-called modular extracorporeal liver support (MELS), was able to support all six patients till transplantation and also improved patients neurological conditions [149].

The flat membrane bioreactor BAL system consists of a multitude stackable membrane modules [151]. Each module can be identified by two oxygen permeable membranes which are distanced between them by a cellular compartment. In this latter, hepatocytes are present in a “sandwich-culture” configuration. In fact, the cells are first seeded on one membrane coated with type I collagen. After hepatocyte adhesion, a second layer of collagen is placed between the cell layer and the other membrane. Patient’s plasma and oxygen are perfused or infused at the surface of the opposite sides of the module. The advantage of this system is surely associated with the stable culture method for the hepatocytes. No clinical trials have been performed so far for this type of device.

In conclusion, hollow-fiber systems, despite their rapid access to clinical trials, possess physical limitations, such as constrained total mass diffusion distances, limited cellular mass capacity, and no uniform cell distributions [148, 152]. On the other hand, the flat sheet design, which is likely one of the most extensively developed, presents the advantage to easily incorporate scaffolds and cells, but its surface area-to-volume ratio is low and it is difficult to scaling up [148, 152].

2.3.2.2. PERFUSED BEDS/SCAFFOLDS BIOREACTORS

Such class of bioreactors revealed an evident potentiality in improving the mass transfer efficiency thanks to the direct perfusion of the adhered hepatocytes with the patient’s blood or plasma. Hepatocytes are usually attached to matrices of various natures. However, the immunological barrier is missing in this configuration [147]. Aspects of certain representative bioreactors are presented here.

The Academic Medical Center (AMC) BAL system likely presents the most studied example of direct perfusion bioreactor. It consists of a nonwoven hydrophilic polyester matrix for cell adhesion and hollow fibers for oxygen supply and a spacer between the matrix [149]. The matrix is spirally wound with longitudinal hollow fibers. Primary porcine hepatocytes ($4.4 \times 10^8 - 2 \times 10^{10}$), attached to the matrix as small aggregates, are in direct contact with the plasma of the patient while receiving optimal oxygen supply. In clinical studies, the AMC-BAL treatment successfully bridged the patients to transplantation, beyond reducing the plasma ammonia and bilirubin concentrations [149].

Another type of direct perfusion bioreactor is the radial flow bioreactor (RFB) BAL system which consists of a woven-nonwoven polyester matrix sandwiched between two polyester screens which prevent hepatocyte leaking out of the bioreactor during plasma perfusion [153]. Primary porcine hepatocytes (200 g), in the form of aggregates, are loaded in the matrix and perfused in radial-flow geometry with the plasma of the patient. Specifically, the plasma flows from the center to the periphery of the module. RFB-BAL treatment, in patients with different stages of liver failure, allowed improvements in the level of encephalopathy and decrease of plasma ammonia and bilirubin concentrations. Furthermore, it supported most of the patients till transplantation [154].

Beyond the enhanced mass transfer, this bioreactor category provides a three-dimensional culture environment which is beneficial for the stabilization of the hepatocytes. By contrary, cells are exposed to shear stress. Additional drawbacks are represented by the no uniform perfusion profiles and the occurrence of membrane pores clotting [148, 152].

2.3.2.3. ENTRAPMENT-BASED BIOREACTORS

This type of bioreactors uses the strategy to encapsulate the hepatocytes in semi-permeable spherical structures, identified with the terms of beads or capsules, which are then inserted in an extracorporeal vertical column. The spherical structures act as anchoring membranes for the cells, providing them with a three-dimensional environment, but also as immunological barrier. The bioreactor designs are broadly grouped in two different configurations, so defined fixed or fluidized bed [147]. In the former, beads are densely packed into the column. It shows some limitations in scaled-up usages, including the formation of preferential flow channels, which results in poor perfusion of a large volume of beads and, therefore, limited mass transfers, but also the exposure of the beads to high shear stresses. Indeed, in the fluidized bed bioreactor, the beads are maintained in continuous, controlled motion. In this manner, the exchanges (plasma-hepatocytes and vice versa) of metabolites are more efficient thanks to an enhanced mass transport. To date, these bioreactor systems have been utilized in *in vivo* animal studies. Some examples of these bioreactors are shortly described below.

The UCLA-BAL system involves the direct hemoperfusion of microencapsulated porcine hepatocytes in a fixed bed bioreactor. Beads were composite membranes made of calcium alginate-polylysins-sodium alginate. The device was used in an animal model of fulminant hepatic failure and provided promising improvements of the survival rate of the animals compared to hollow-fiber based BAL devices [155].

With a configuration similar to that of the UCLA bioreactor, another entrapment-based bioreactor utilizes alginate-entrapped porcine hepatocytes spheroids (that are spherical aggregates) instead of single hepatocytes at the aim of ameliorating the performance of the hepatic functions. Hepatocyte spheroids are pre-formed in culture within 20h and, then, immobilized in calcium-alginate beads. This system was only evaluated at anhepatic pigs [149]. In our laboratory, an alternative type of entrapment-based bioreactor has been developed about 20 years ago (**Figure 13**). It is a fluidized bed bioreactor containing calcium-alginate microencapsulated hepatocytes designed to be perfused by the patient plasma. The beads are expanded till a maximal height in the bioreactor, thus, they are maintained in continuous motion in a regular loop from the bottom to the maximal height. Such movement derives from two combined events: the push of the beads from the bottom to the top of the bioreactor due to the plasma perfusion, and the tendency of the beads to sedimentate on the bottom part of the bioreactor under gravity action because of their higher density compared to that of the plasma [156]. Studies are still going on for the purpose of refining its operation parameters as well as ameliorating the preservation of the cellular component functions. However, some *in vivo* animal studies have been already performed to investigate the safety of the system in large anhepatic rams [157]. In addition to the above mentioned potentiality of improving the mass transfers (fluidized bed bioreactor), these systems provide uniform environment and are easy to scale-up [148, 152]. On the contrary, issues may be linked to the degradation of the microbeads for long-term usage with consequent dispersion of the cell in the patient plasma, and to the cell exposition to shear stresses, even though the bead may provide a mechanical protection to the cells [147, 148].

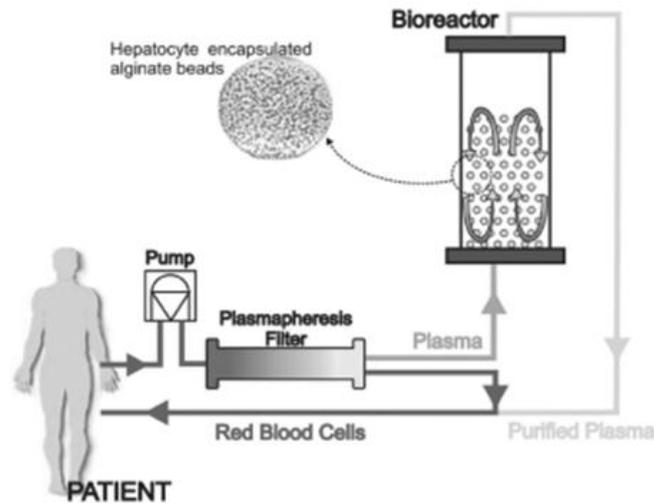


Figure 13. Schematic representation of the fluidized bed bioreactor, containing the alginate beads of hepatocytes maintained in continuous motion, and the corresponding circuit to which the patient is connected during the treatment [158].

2.3.3. CELLULAR COMPONENT AS CRITICAL ISSUE OF THE BIOARTIFICIAL DEVICES

Although pre-clinical and clinical trials highlighted the promising role of the BAL systems [159, 160] in supplying patients in acute/fulminant liver failure and in bridging them till transplantation, these devices have not been introduced yet in the routine care. Indeed, none of these clinical studies showed a significant effect of the BAL treatment on patient survival considered as the major endpoint.

Primary human hepatocytes (PHH) represent the ideal cell type to use in BAL devices. The definition of a suitable, sufficient mass of hepatocytes is a fundamental parameter for the adequate replacement of the hepatic functions and, consequently, the correct performance of these systems. To date, different numbers have been proposed by several research groups. Based on safe surgical resections, common opinions converge on the fact that survival is possible with a minimum of the 20% of liver mass with optimal functionality, which corresponds to about 200 g or 20×10^9 hepatocytes [153, 161]. However, considering that the main source of PHH is discarded donor livers, which are inappropriate for transplantation and often deriving from pathological conditions, these cells do not have optimal functionalities. In addition, hepatocytes functions are subject to a rapid drop in *in vitro* environments, like that of the bioreactor. Such circumstances imply to favor a higher mass of hepatocytes (25-30%) for BAL applications. Unfortunately, their availability is very low, equivalently to the scarcity of liver donors for transplantation, thereby, this substantial cell demand needs to be alternatively addressed. The *in vitro* proliferative inability of human, but more in general primary, hepatocytes does not provide any solution to the situation. To overcome these obstacles, a variety of strategies have been proposed and attempted. Two solution lines can be broadly distinguished. A first one aims to resolve the issue of low availability of human primary hepatocytes by seeking alternative cell sources. The other intends to maximize and preserve the functionalities of the isolated human primary hepatocytes in culture milieu.

Regarding the alternative cellular sources, animal hepatocytes have been largely adopted in bioreactors. In particular, porcine hepatocytes have been utilized in the majority of the clinical trials, as deductible from the previous paragraphs. This cell type presents the advantage to be available in large quantities and obtainable on demand, and also to possess

high functional levels. However, as already discussed, porcine (and largely animal) hepatocytes can lead to immunological problems that are associated with the production of xenogenic proteins, as well as risk of zoonosis or virus transmission. Even though these issues are not expected to occur in treatments that last less than 5-6 days, many European countries prohibit their clinical application [153].

Cell lines have been also used in BAL systems as an alternative for PHH. Several cell lines have been established and one type (C3A), derived from hepatic tumors, has been used in clinical trials. Despite their large availability and proliferative potentiality, functionality and safety of these cells remain questionable aspects of their clinical usage. In fact, C3A cell lines are not able to perform the wide spectrum of the human hepatocyte functions (such as ammonia removal [153]) and they can be the origin of transmission of oncogenic substances into the patient's circulation [148, 153, 162].

Other potential cellular sources are being considered for liver support systems, including adult and embryonic stem cells, fetal hepatoblasts, oval cells and stem cell-derived hepatocyte-like cells [148, 162]. Because of ethical issues, poor understanding of differentiation processes, and lack of standardized protocols, their use in actual treatments is not possible yet and further investigations are being done.

The rationale of the second solution line is to provide the hepatocytes with some cues that recreate the *in vivo* microenvironment [148, 162]. To this end, several approaches have been proposed and mostly involved manipulation of the media composition and the environment, or promotion of cell-cell interactions [23, 148, 162]. Although it has been demonstrated that supplementation of the culture media with hormones or other components (for instance, dexamethasone) contributes to stabilize HEP survival and functionality, this approach cannot be transferred to actual BAL applications. On the contrary, changes of the environment, by providing a three-dimensional configuration to the cells via the introduction of ECM components and/or the establishment of self-assembled aggregates (e.g. spheroids), as well as inclusion of NPCs, for the promotion of interactions with HEPs in a co-culture setting, are applicable to BAL designs. Both these strategies have been extensively investigated in more fundamental studies, which testify their beneficial effects in stabilizing and maintaining the HEP functions [11, 23, 148, 162], and in some *in vitro* BAL applications, as in that case reporting the introduction of porcine HEPs and NPCs in a collagen-sandwich configuration inside a flat membrane bioreactor [163]. Because of the incomplete understanding and management of these elements, it has been impossible to include them in clinical studies.

Scope of the next paragraphs is to go through the different publications that report co-culture strategies in the hepatic context in order to summarize the principal outcomes and infer what improvements still remain to be taken into account and what condition may most promisingly be translated into future clinical BAL application.

2.3.3.1. CO-CULTURE: PROMISING STRATEGY FOR HEPATOCYTE STABILIZATION IN LIVER TISSUE ENGINEERING

The term "co-culture" refers to a culture setting of multiple, distinct cell types cultivated together within the same environment. Co-culture techniques have been utilized in disparate fields for several purposes, such as fundamental investigations of biological complex mechanisms occurring between different cell populations in the organs, which are otherwise inaccessible, or creation of a milieu similar to the nature system in order to generate artificial organs for more practical medical applications.

Co-culture systems were introduced in the field of biological research at the very end of 1970s with the general scope to study cell-cell communications [164]. Simultaneously, co-

culture also appeared in the experimental area of the hepatic research as different option to the two rooted strategies of the time (i.e. medium composition and culture substrates) to maintain HEP survival *in vitro*. Early studies involved primary rodent HEPs and, predominantly, fibroblast cell lines in simple co-culture systems, designed by mixing cells together and seeding them on collagen-coated or bare culture dishes, in order to examine the retention of biochemical and morphological features of the hepatic parenchymal cells [165] for the final purpose of employing this pattern in cytotoxicity tests [166], as alternative to the liver microsomes used at that time, or in carcinogenesis investigations [165, 167]. However, the differentiated state of the cultured cells was evaluated only qualitatively in these models and the hepatic specific functions were scarcely considered. In 1983, Guguen-Guillouzo et al. first demonstrated the effectiveness of a co-culture system in enhancing hepatic functionality. In this report, rat primary HEPs exhibited long-term secretion of albumin (over 2 weeks) when co-cultured in close and direct contact with rat liver epithelial cell lines in a mixed, two-dimensional (2D) manner [168]. The authors speculated about the critical role of cell-cell interactions not only in modulating levels of albumin production, but also in synthesizing ECM components (fibronectin and various collagen types) that in turn may have contributed in the maintenance of the protein secretion. Beyond the complexity of the co-culture systems and the difficulty in explaining the mechanisms involved, the outcomes of this first study were promising and made researchers trustful in pursuing the co-culture approach in the liver domain. Since then, the number of publications - focused on improving and preserving HEP activities in culture – increased; this aspect may be also due to the pressing demand of feasible solutions responding to the shortage of transplantable livers and which implied the preservation of the HEPs in culture environment, such as that of the bioreactor in the BAL devices.

The establishment of a co-culture system engages several critical parameters and only their correct definition, although is not easy and requires accurate analysis, can lead cells toward more physiological interplays and responses. From the scrutiny of a myriad of proposed co-culture models, some elements are identifiable as paramount criteria for the formulation of a promising co-culture system; these include the choice of the cellular populations, the ratio between the different cell types, and the design of the culture environment. The coordination of all these factors regulates cellular events through the promotion of soluble signaling, physical cell-cell and cell-matrix interactions. Aim of this review is to examine such aspects in order to get insight about their role in maintaining HEP functions *in vitro*.

2.3.3.1.1. MECHANISMS INVOLVED IN THE MODULATION OF HEP BEHAVIOR BY CO-CULTIVATION

Numerous studies have been carried out to understand the mechanisms underlying the regulation of HEP functions in case of co-cultivation with other cells. Yet, a clear scheme still remains to be drawn down. As stated by Bathia et al. [169], the influence of co-culture on HEP activities has been measured using “all or nothing”-type outcomes. In fact, early studies typically compared i) HEPs in co-culture to HEPs cultured alone, ii) HEPs separately cultured from the second population (e.g. by the porous membrane of a transwell), or iii) HEPs exposed to media “conditioned” by the second cell type [169]. By means of these culture settings, investigators especially tried to infer the role of cell-cell interactions and/or that of soluble factors. However, hepatocellular functionality in co-cultures may be simultaneously influenced by the combination of multiple elements [169, 170], including three cellular variables (homotypic HEP interactions, heterotypic interface, and homotypic interplays of the nonhepatocyte populations) as well as secretory aspects (soluble signaling molecules or

insoluble ECM components). Already in 1988, Goulet et al. [171] identified both soluble factors and cell contacts as distinctive frames of a bigger picture. Accordingly, the production of soluble factors was considered as a part of the mechanism involved in the stabilization/enhancement of the HEP functionalities, whereas, the cell contacts were claimed as necessary for the full expression of the cell help evinced in co-cultures. However, disparate groups highlighted the importance of the former rather than the latter component. Different findings may be linked to the type of cell used in addition to the HEPs in the co-culture system. In regards to this possibility, Otsuka and coworkers [172] concluded that direct heterotypic interactions were solely beneficial to HEP performance in presence of endothelial cells, in contrast with a double favorable effect of both direct contacts and soluble factors when HEPs were co-cultured with fibroblasts. In addition, the relative position of different cell populations in the same co-culture system may be important for the maintenance of the *in vitro* HEP activities contributing to the establishment of cell-cell interactions or the secretion of soluble factors [173]. The paramount role of the cell-cell interactions in enhancing and prolonging the functional maintenance of the primary HEPs was amply reported in case of co-cultures with the fibroblasts [174-177]. Cho et al. [178] localized high levels of albumin staining uniquely in regions of increased heterotypic interface in their defined micropatterned cellular islands. In line with this result, Seo and collaborators [174] suggested that the enhancement of the hepatic functions was due to the maximization of the cell-cell interactions between HEPs and fibroblasts in a co-culture system of a 3D alginate/chitosan sponge. This functional improvement was directly associated with a more rapid expression of connexin 32 and E-cadherin. On the other hand, Khetani et al. [176] hypothesized that cell-cell interaction was likely to be multifactorial (requiring, for instance, ECM deposition and direct contact). In their study, in fact, they identified decorin, a major liver proteoglycan, and N-cadherin, a cell surface protein, as two probable candidates involved in the mechanism of HEP-fibroblast direct communication and, consequently, enhancement of the hepatic functionalities. Similarly, Underhill et al. [175] suggested a combinatorial impact of paracrine and cell contact-mediated signals provided by the fibroblasts on the long-term maintenance of the HEP functions in their randomly organized co-culture system. Complementary engagement of both cell-cell contacts and secreted signals was testified in case of co-cultures of endothelial cell with HEPs. Harimoto et al. [179] suggested that the direct contacts between HEPs and endothelial cells were primarily responsible for the longer preservation of the hepatic functions, supporting, in addition, the idea that insoluble factors were potential mediators of the established interactions. Jindal and coworkers [180] demonstrated a significant enhancement of hepatic functions (albumin production) either in presence or in absence of heterotypic interactions between HEPs and endothelial cells. A recent study [181] confirmed somehow these previous findings. In this case, the HEP-endothelial cell interactions appeared to be fundamental not only in stabilizing and ameliorating some hepatic functions (albumin production and CYP activity) but also in preserving the phenotypes of both co-cultured populations. However, the authors did not exclude the beneficial action of released soluble factors in this context. Such analogous outcomes may be connected to the set-up of similar co-culture approaches. In all these researches, in fact, a layered cell based co-culture technique was adopted which may have allowed the endothelial cells to intrinsically synthesize their own ECM (insoluble factors) [182] providing the HEPs with supplemental *in vivo*-like cues. In addition, HEPs may be also stimulated to produce ECM components. Proline was identified by Jindal et al. [180] as key soluble factor secreted by endothelial cells and involved in the promotion of collagen synthesis by HEPs as well as the improvement of hepatic protein secretion. This amino-acid seemed to act, at least in part, at the

transcriptional level. An early study [183] already suggested that part of the mechanism implicated in long-term functional stability of the HEPs in co-culture with a different cell type, namely the epithelial cells, was of transcriptional origin. The authors hypothesized that an undefined factor, provided by the epithelial cells, could be involved in the transcription of liver-specific genes in the HEPs. Other investigations pinpointed the involvement of multiple signals for the preservation of hepatic functions in case of co-culture between HEPs and HSCs. Nevertheless, there is common opinion that HSC secreted soluble factors, including cytokines and growth factors (such as the hepatic growth factor), play a pivotal role here [184-186]. Interestingly, Krause et al. [187] further sustained the potentiality of cell membrane-bound signals, transferred between the different cell populations (e.g. the exchange of lipids), in the maintenance of the HEP differentiation and functionality. Soluble factors represent the principal responsible of the cellular cross-talk between HEPs and KCs. Under specific co-culture conditions, certain KC released mediators, generally of pro-inflammatory identity, initiate a cascade of events that lead to the depression of biotransformation capacities and protein production in the HEPs [188-190] reminding the acute-phase response of the liver. According to Peterson et al. [190], the decrease of CYP450 enzyme activity indirectly resulted from the phagocytic action of the KCs, during which these cells may secrete an unknown factor. Hoebe and coworkers [191] asserted that cell-cell interactions could increase the production of cytokines and amplified the effects on the HEPs. In line with this statement, Zinchenko et al. [192] sustained that direct cell contacts could inhibit HEP protein synthesis in a ratio dependent manner. When KCs outnumbered the HEPs, the established interactions augmented. In this case, HEPs may release a soluble factor that, in turn, may induce the KC activation and augment their secretory capability. Activated KCs likely increased their phagocytic activity impacting the HEP functionalities through those messengers secreted during phagocytosis.

Despite the reported assumptions regarding the involvement of the direct cell interactions and/or the indirect communication by exchange of soluble factors, co-culture influences the stabilization of the hepatic functions via a variety of ways of which the specific mechanisms remain unclear.

2.3.3.1.2. CELL CHOICE AND CO-CULTURE RATIO

Some cells cannot easily be monocultured *in vitro* and/or do not exhibit desired *in vivo* physiological behaviors. In co-culture, the cultivation success of these cells can be improved by the presence of another or multiple cell populations [193]. The used cell types in a co-culture are usually defined *target* and *assisting* (alternatively, supporting or feeder) cells [194]. The former are those responsible for the functions that must be recovered in the *in vitro* systems (i.e. the HEPs in the hepatic domain). Whilst, assisting cells help the target cells to perform and preserve their activities by constantly responding to their requests. The choice of the assisting cell type is essential for long-term efficacy of the target cells.

In the specific case of the BAL devices, assisting cells co-cultured with HEPs can ideally allow the reduction of the high requested hepatic cell mass by enhancing HEP functions, provide an independent therapeutic benefit to disease, and ameliorate the preservability of HEP storage before the treatment [195]. Few sporadic studies reported the inclusion of supporting cells in BAL systems likely because there is not unanimous opinion on their optimal choice yet and much still remains to be elucidated. On the contrary, a broad variety of assisting cell types have been employed in the “bench” hepatic researches with the intent of creating transferable liver models for predicting drug metabolism/clearance and for BAL application. First argument of the choice relies on the source of the assisting cells. An open

question evolves from this point: whether cells derived or not from the liver can differently impact on the preservation of the hepatic functions. A further distinction must be taken into account concerning the utilization of primary cells or cell lines. Already in 1983, Guillouzo et al. supposed that cooperation between HEPs and assisting cells was tissue-dependent justifying the reduced survival and functionality of the HEPs in co-culture models that used cells of non-hepatic origin [168]. In contrast to this statement, Morin and collaborators provided evidence of the nonspecificity of the cellular interactions with respect to tissue, organ and species for the stabilization of the HEPs. In their studies, primary rat HEPs extended their viability and preserved the activity of albumin secretion in presence of pulmonary endothelial cell lines, bovine aorta endothelial cells, and human foreskin fibroblasts [171, 196]. Due to these controversial findings and the lack of clear explanations, supporting cells either derived or not from the liver were object of investigation in the following years (**Table 3**).

Table 3. Non- and derived liver cells (primary (P), subcultured (SC), and cell lines (CL)) used in co-culture with mature HEPs.

DERIVED LIVER CELLS		NON-DERIVED LIVER CELLS		RATIONALE
TYPE	SOURCE	TYPE	SOURCE	
<i>Hepatic Stellate Cells</i> [184-187, 197-203]	❖ Human (CL) ❖ Rat (P, CL)	<i>Fibroblasts</i> [169, 172-178, 195, 204-214]	❖ Human derma (P, SC) ❖ Mouse embryo (P, SC, CL)	Production of extracellular matrix components and, in the case of hepatic stellate cell, release of growth factors (e.g. hepatocyte growth factor) fundamental for hepatocyte support
<i>Liver sinusoidal endothelial cells</i> [181, 198, 215-219]	❖ Human (P) ❖ Rat (P)	<i>Endothelial cells</i> [172, 179, 180, 182, 202, 205, 217, 220-225]	❖ Human umbilical vein and aorta (P, SC, CL) ❖ Bovine aorta, carotid artery, and pulmonary microvessels (P, SC, CL) ❖ Rat prostate (CL) and heart microvessels (P)	Support of the performance of various metabolism functionalities (such as that of the lipids) as well as the response to inflammatory and toxicant stimuli
<i>Kupffer Cells</i> [188-191, 214, 216, 219, 226-229]	❖ Human (P, SC) ❖ Rat (P, SC) ❖ Porcine (P, SC)	<i>Monocytes</i> [223]	❖ Human leukemic lymphoma (CL)	Mediation of inflammation toxicity and regulation of hepatic specific functions, such as acute-phase response, by release of cytokines
<i>Epithelial Cells</i> [183, 230-233]	❖ Human (P, SC) ❖ Rat (P, SC)	<i>Epithelial cells</i> [234, 235]	❖ Canine kidney (CL) ❖ Monkey kidney (CL) ❖ Human embryonic lung (CL)	Establishment of hepatic mechanisms dependent on cellular interactions that require peculiar cellular membrane receptors present in biliary epithelial cells
<i>Non-parenchymal cells (whole fraction)</i> [175, 216, 236-245]	❖ Rat (P)			Closely approaching the <i>in vivo</i> complexity of the liver. Support of a combination of hepatic functions
		<i>Mesenchymal stem cells</i> [195, 246-248]	❖ Human/ Porcine/Rat Bone Marrow (P) ❖ Human adipose tissue (P)	Production of crucial cytokines and endogenous extracellular matrix proteins that sustain the hepatocyte functions

❖ PRIMARY DERIVED LIVER CELLS

The rationale behind the selection of primary derived liver populations is to provide an environment that resembles that of the liver firstly considering its cellular composition. Several primary derived liver cell types have been employed in co-culture models. Biliary epithelial cells represented a possible choice, although their use has not been very frequent. Auth and co-workers [230] utilized only this cell type in co-culture studies implying the involvement of the biliary epithelial cells in hepatic mechanisms dependent on cellular interactions requiring particular cellular membrane receptors. However, the hepatic sinusoidal NPCs were principally chosen in this context. The reason of this pick relies on the support given by these cells in the service of the HEPs through the release of mediators

(such as cytokines, growth factors, hormones, active oxygen derivatives) or direct cooperation that seem to profoundly affect the hepatic functions in *in vivo* physiological and pathological circumstances. However, less is described about the contribution of these hepatic cells under normal conditions [10]. Besides these collaborative aspects, the expectation in utilizing specific NPCs in co-culture with HEPs is to improve the completeness of the co-culture system by incorporating functional activities that are principally attributable to the NPCs. In principle, LSECs should increase the specificity of the system by ameliorating the performance of various metabolism functionalities (such as that of the lipids) as well as the response to inflammatory and toxicant stimulus [181, 249]. KCs should especially be mediators of inflammation toxicity [219] and responsible for the regulation of the acute-phase response by way of the release of cytokines [226]. Whilst, HSCs, natural companions of the HEPs [185] with which establish physical and chemical contacts *in vivo* [250], should predominantly see to the production of matrix [249] and provide HEPs with specific growth factors (e.g. hepatocyte growth factor) [186]. LSECs, KCs, or HSCs were generally obtained from rat or, in minority, porcine livers and separated from the rest of the liver cells. Following isolation, the primary assisting cell type was either immediately allocated in the co-culture model or monocultured for an acclimation/recovery period before their usage in co-culture with HEPs. Although it has been claimed that pre-culture of NPCs is important to avoid their activation due to the stress-inducing culture conditions (as in the case of the KCs) [11], it is extensively known that, in spite of the presence of ECM coating or supplemented medium, LSECs, KCs, and HSCs rapidly dedifferentiate when cultured in monolayer (as for instance the acquisition of a myofibroblast phenotype by the HSC) losing their peculiar features (e.g. the disappearance of fenestrae in LSECs) and capability in sustaining the HEPs in a physiological manner [186]. Furthermore, this pre-culture approach implied the creation of subpopulations of the initial isolated cell type and, consequently, the sacrifice of different and multiple animals for the setting of a sole co-culture system. In alternative to the co-culture of single distinctive populations, many groups concentrated on cultivating the whole fraction of NPCs [212, 216, 236-242, 244] extracted from the same liver used for the isolation of the HEPs. By applying this mix of hepatic cells, the authors intended to more closely approach the actual situation of the organ thanks to the establishment of a hierarchical organization [236] in which the several cell types autonomously arranged together in the most proper manner allowing the setup of multiple direct or indirect interplays between themselves and the HEPs but also the synthesis of various ECM components [237, 241]. Despite the promising tissue-like configuration, the culture of the entire NPC fraction may present some limitations concerning the lack of control of its composition and, accordingly, the impossibility to setup comparable co-culture models presenting the same cell seeding characteristics.

It is conceivable that the use of primary liver derived cells is susceptible to limiting aspects. These include practical facets associated with the complex isolation procedures of the different cell populations that, beyond requesting manual technical skills and the availability of suitable lab equipment, are time-consuming and low yield methods. On the other hand, their commercial availability is scarce and costly. Additional drawbacks are related to the difficulty of culturing these cell types. However, there is evidence that co-culture can be also beneficial for the phenotypical maintenance of these NPC types, even though the addition of peculiar growth factors may be indispensable. Nevertheless, their long-term preservation remains challenging.

For these reasons, the research of different cell choices became mandatory.

❖ PRIMARY NON-DERIVED LIVER CELLS

One possible option was to use primary cells obtained from non-hepatic organs. This decision may have taken advantage of simpler isolation procedures as well as the selection of less demanding cell types. Based on the crucial influence that endothelial cells perform on the HEPs, these cells were the main non-derived liver population to be involved in hepatic co-culture models. Disparate sources of endothelial cells were utilized, including the human umbilical cord (HUVECs) [222, 224, 225], the bovine carotid artery (BCAECs) [182] and aorta (BAECs) [220], or the rat heart microvessels (RHMECs) [180]. In alternative to the endothelial cells, bone marrow mesenchymal stem cells (MSCs), of human or rat origin, were depicted as suitable candidates to introduce in co-culture system of primary HEPs [246-248]. This fact was especially associated with several beneficial abilities of the MSCs, such as their potentiality to regulate cell growth and differentiation by secreting various soluble factors and ECM proteins [247].

❖ CELL LINES

In complete opposition to the employment of primary cells, a huge number of co-culture models were defined by the presence of cell lines. The advantages of using these cells relied on their ready availability, simplicity to be cultured, potential easy growth, long lifespan, and stable phenotype [173, 176, 184, 234]. Nevertheless, some culture issues, such as the uncontrolled overgrowth, the incompatibility with the HEPs, and the incomplete tissue-specific gene expression, have been correlated to the cell lines. Utilized cell lines were prepared from primary non-parenchymal liver-derived cells (including the immortalized rat liver stellate cell lines, HSC-T6 [250] or HSC-Li [184], and the mouse Kupffer cell line, KC13-2 [249]) or obtained from other organs (for instance the human embryonic lung L-132 cell lines, the canine kidney epithelial MDCK cell lines, the monkey kidney MS cell line [234]). Among these, fibroblasts were mainly preferred because of their crucial role in the synthesis of ECM and production of hepatocyte growth factor [177], although their usage did not respect the physiological hepatic pattern as the fibroblasts are not in physical contact with the HEPs in native liver tissues [205]. The murine embryonic fibroblast cell lines (3T3-J2, NIH-3T3) were extensively adopted in different co-culture configurations and provided the most successful culture conditions for the HEPs [249].

❖ CO-CULTURE CONDITIONS AND RATIO

The choice of the suitable supporting cell holds a fundamental task in the set-up of a co-culture system; however, it requires to be combined to other parameters. The liver is a complex organ constituted by a multitude of cell populations inhabiting the tissue in precise fixed densities and mutually collaborating for the correct performance of the hepatic activities. Taking into account such physiological complexity of the tissue, it may be important to include several cell types in the same co-culture milieu and respect their original relative numbers at the seeding time? Although the answer is still matter of debate, some authors tried to better explore these conditions. Kostadinova et al. [244] supported the idea that a co-culture system lacking in various hepatic NPC populations does not recapitulate all the important functions of the liver. However, a critical aspect in this context is likely represented by the difficulty in sustaining multiple cell types in a sole culture microenvironment. Therefore, most of the defined co-culture systems restricted their investigation to only one assisting cell type in addition to the HEPs which were, generally, cultivated in a ratio of 1:1. Few other studies reported more sophisticated co-culture concepts. Beyond the systems including the whole NPC fraction, some groups cultured two or three feeder cell populations with HEPs by controlling their seeding densities. The

majority of tri-culture models was established between 2011 and 2014 and included two assisting cell types in addition to the HEPs. Kasuya and coworkers [202] chose rat HSCs and (bovine pulmonary microvascular) endothelial cells because of their pivotal role in cell-cell communication in the liver in order to recreate an *in vivo*-like structure. Similarly, Jeong et al. [198] employed primary subcultured rat HSCs and LSECs for the creation of a *in vivo* mimetic micro liver tissue in which cell signaling, cell-cell interactions and morphogenesis of the normal liver tissue were probably mimicked. With the identical intent to construct an *in vitro* model for the establishment of cell-cell and cell-ECM communications similar to native liver structure, Liu and collaborators [205] identified the mouse embryonic NIH3T3 fibroblast cell lines and human umbilical vein endothelial cell lines as appropriate assisting cell populations for the co-culture with the HEPs. Whereas, Messner et al. [219] incorporated KCs and LSECs at the purpose of providing the HEPs with diffusible growth factors and cytokines while establishing heterotypic cell-cell contacts with the final goal to define an *in vitro* inflammation-mediated toxicity model. Recently, Prodanov et al. [223] presented a co-culture system showing facets of human liver physiology and functions. This system included three different human cell lines in addition to the HEPs as substitutes of the hepatic NPC populations, namely, the human umbilical vein cell line (EA.hy926), the human hepatic stellate cell lines (LX-2), and the human leukemic monocyte lymphoma cell lines (U937), in order to closely approach the actual cellular composition of the organ. Only a minority of publications included preliminary analysis focused on establishing an optimal co-culture ratio of HEPs and assisting cell type(s). Cells co-cultured according to diverse ratios can differently arrange in the space of the system and, consequently, form interactions with distinct degrees of complexity affecting the cell ability of releasing soluble factors, depositing ECM matrix, and, thereby, sustaining the HEPs functionalities. There are disparate viewpoints within the literature regarding the optimum ratio. These differences may rely on the choice of the employed feeder cell type(s) and of the technique of co-culture as well as the pick of the functional assessment. Considering similar (spheroidal) co-culture systems, it can be observed that five-fold excess number of sinusoidal endothelial cells [251] or of (whole fraction) NPCs [242] did not provide the highest level of albumin production which was rather attained when the mixing ratio of HEPs and supporting cell population was 1:1. On the contrary, a surplus (10 times higher) of HEPs over the human adipose mesenchymal stem cell number generated the greatest albumin synthesis [246]. However, when murine embryonic fibroblasts outnumbered the HEPs, the level of albumin secretion augmented [169, 177]. These cited outcomes may lead us to conclude that the choice of the assisting cell type is deeply implied in the definition of a co-culture ratio. Yet, different *in vitro* liver activities may be beneficially affected by other diverse optimal ratios. For instance, Chia et al. reported an augmented urea production when the HEPs were cultivated with murine embryonic fibroblasts in equal densities (in a ratio of 1:1) in a (spheroidal) co-culture system. This result appears to be in contradiction with the previous finding in which the improvement of albumin secretion occurred in presence of a higher seeding density of fibroblasts over the HEPs. Although the several co-culture systems proposed in literature are difficult to compare due to the diverse culture conditions (e.g. the medium composition), this analysis indicates that a specific liver function may show a tendency to be stabilize by a precise cellular ratio between HEPs and assisting cells as well as to be dependent on the supporting cell type of defined origin. Finally, these examples also suggest that the ratio of HEPs to a specific NPC type in the natural liver does not exactly coincide with the relative cell proportion that provides the optimal outcomes in the co-culture system [192].

2.3.3.1.3. DESIGN OF AN ARTIFICIAL CO-CULTURE MICROENVIRONMENT: DIFFERENT TECHNIQUES

Depending on the research purpose, co-culture systems have been set up on the base of specific methods. A considerable amount of co-cultures, especially related to initial studies, utilized a “randomly distributes” approach, in which the assisting cell population(s) - generally only one additional type - was simply mixed with the HEPs and cultivated together on coated or bare culture dishes in a two-dimensional manner. Although these investigations primarily aimed to elucidate how the functions of a model tissue were affected by cell-cell interactions [169], the major limitation of this co-culture method was the lack of control of the direct contacts between the cells. Here, the cell interactions, as reported by Bathia et al. [169], are highly variable between each co-culture and depend on the seeding densities, cell aggregation and migration, as well as the cell ratio between the different populations. Such facets lead to disequilibrium in the establishment of the cell interactions, as, for instance, the promotion of homo- upon heterotypic contacts or vice versa. In aid to these drawbacks, some other co-culture techniques have been attempted.

❖ MICROFABRICATION AND MICROPATTERNING

Microfabrication provided feasible advantages. Various patterning technologies were developed for the purpose of designing more controlled local contacts between neighboring cells [252] so that to hold constant the heterotypic interfaces [169] and, thus, better mimicking the *in vivo* milieu. The origin of cell patterning dated back to the 1960s and is associated to the works of Carter [253, 254]. Yet, the major breakthrough occurred in the late 1980s when Kleinfeld and coworkers applied the photolithography process, utilized only in the microelectronics industry at that time, in the biology field [255]. The creation of a specific pattern via photolithography takes place by irradiating a substrate, coated with a photoresist, through a stencil-like photomask, possessing the geometrical pattern that has to be transferred to the substrate. Following to irradiation, the photoresist results altered and becomes soluble or insoluble in specific solvents. Therefore, parts of the photoresist can be washed away in a solvent permitting to the pattern to come out on the substrate. Later in '90s, Whitesides and collaborators developed another patterning technique, so-called soft lithography [256]. This latter, presented as a non-photolithographic strategy, uses photolithographic technology only to design a patterned, soft, elastic material (usually the poly(dimethylsiloxane), PDMS) in the form of a stamp, mold, or mask which is in turn utilized to pattern a substrate [252]. Several methods exploit the soft lithography principles to generate patterns and structures showing peculiar features, such as the presence of stamped proteins onto a surface. Microfluidics is included among these; it consists in sealing the prepared flexible mold or stamp against a surface to form microchannels through which a fluid can be drawn by capillary forces. These methodologies remained initially limited to the study of single cell type. The patterning of cells in co-culture posed some difficulties, including the exposure of the first cell population, patterned on a substrate, to severe chemical or physical conditions while patterning the second cell type on the same substrate [252]. Pattern alignment represented another challenging aspect as the second cell type must be precisely localized in specific regions of the substrate relative to the first population in order to control the interactions between them [252]. The first study of patterned co-culture was published by Bhatia and colleagues in 1997 [208]. Here, the authors provided a method for generating two-dimensional, anisotropic surfaces capable of organizing two different cell types (HEPs and fibroblasts) in discrete spatial locations (**Figure 14**).

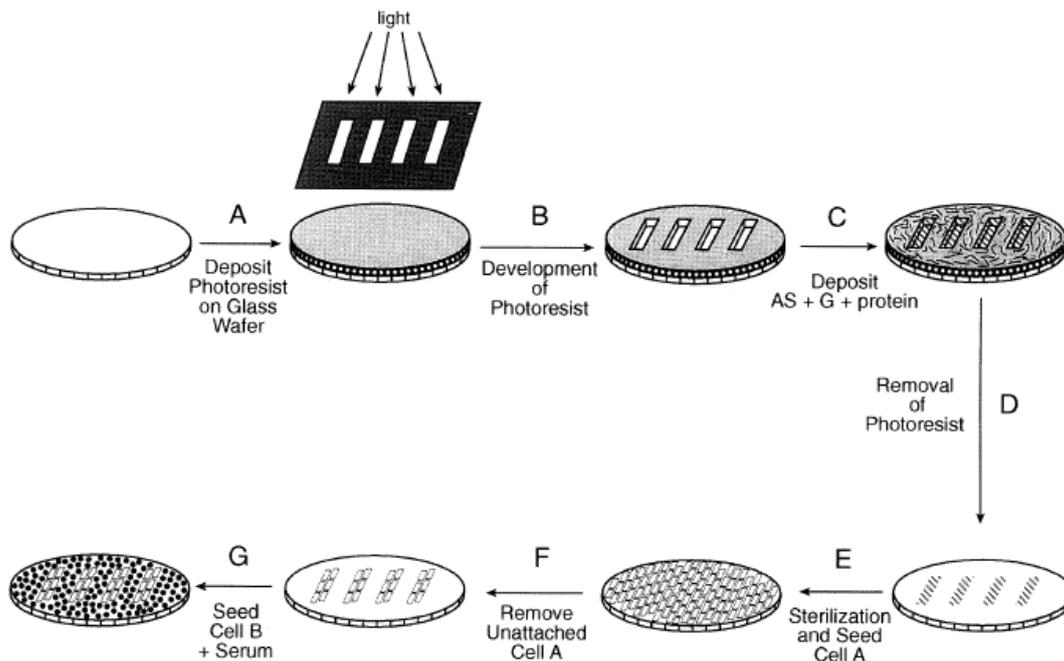


Figure 14. Representative scheme of the method of co-culture patterning proposed by Bathia et al [208].

Photolithography was used to pattern biomolecules on a glass substrate. Cell-adhesive proteins (collagen) were immobilized on specific micropatterned regions of the substrate, whereas, bovine serum albumin (BSA) was bound to the glass background resulting non-adhesive. HEPs were firstly seeded on the substrate in serum-free media remaining attached only to the collagen-patterned areas. Indeed, fibroblasts were inoculated, as second cell population, in presence of serum so that they could easily adhere to the BSA non-patterned zones of the substrate. This process revealed the potentiality to selectively distribute the diverse cell populations on a substrate according to the original pattern and, consequently, to vary initial heterotypic interactions while preserving the ratio of cell populations in culture. Control of the cell-cell interactions appeared markedly enhanced in this micropatterned system compared to that achieved in “randomly distributed” co-cultures. Later, Bathia et al. also demonstrated the strength of this technology in modulating the HEP functions (albumin and urea production) by playing on the configuration of the micropatterned areas as well as on the seeding number of the fibroblasts [169]. To wit, when HEP islands, of fixed diameter, were patterned to a reduced distance between each other on the substrate (implying a reduction of the total patterned surface area) and surrounded by fibroblasts, the hepatic activities improved as a function of the increased number of inoculated fibroblasts, present in excess over the HEPs. These outcomes mainly resulted from the vicinity of fibroblasts to HEP zones [257] and, thereby, from the augmentation of the heterotypic interfaces which, according to Bathia et al., may allow the achievement of supraphysiologic hepatocellular functions *in vitro* and the consequential diminution of the cell mass necessary in the BAL devices. These findings were further confirmed by following investigations. Zinchenko and colleagues [229] established micropatterned co-cultures of HEPs and KCs by taking advantage of the soft lithography technique. In this study, a PDMS stencil was fabricated as a stamp of a micropatterned wafer, this latter realized by means of traditional photolithography procedures, and used as substrate for cell culture. HEPs were firstly seeded in circular collagen-coated patterns of the PDMS stencil, whereas, KCs were later inoculated and adhered to the remaining BSA-coated

regions. In such configuration, hepatic functions (albumin and urea production) were highly increased compared to random co-cultures and the HEPs maintained their viability beyond seven culture days likely thanks to the support given by the KCs. Here, the optimal functional conditions were defined by a dominant presence of HEPs over the KCs. Concerning these patterned co-culture systems, Cho and coworkers [178] proved that the hepatic functionalities could be further improved by increasing the heterotypic contact area between the two cell types. An alternative micropatterning technique, in which the HEPs were patterned on micropatterned fibroblast feeder layers using microfabricated PDMS stencils, produced the best functional performance of the HEPs compared to that obtained when the cells were patterned in neighboring areas confining the heterotypic interface at the edge of the patterned HEP islands.

Despite the promising features of these reported techniques in controlling cell-cell interactions and modulating the hepatic functions, some shortcomings were identified [192]. The glass modification method presents restrictions connected to the photolithographic procedure; in fact, this latter has several limitations when applied to curved, nonplanar surfaces [210]. The seeding of the second cell population poses additional problems: since it occurs directly on the surface of the substrate, the risk of covering also the HEP patterned areas must be taken into account. Furthermore, the harmful materials involved in this technique make the sterilization procedures critical. On the other side, PDMS stencils, nonetheless their fabrication does not involve the usage of injurious substances, possess some physical features (thickness and ratio aspect) that make them fragile and difficult to handle.

❖ LAYER-BY-LAYER AND SANDWICH TECHNIQUES

One potential disadvantage of these types of micropatterned co-cultures is that the cells are generally patterned on a flat surface and form outspread two-dimensional monolayers [207]. In order to refine the level of complexity of the co-culture systems, a three-dimensional component was differently contemplated in several models with the intent of enhancing the co-culture efficacy. In 1991, Decher developed the layer-by-layer assembly technique [258] that permitted to construct polymeric thin films, termed “polyelectrolyte multilayers” (PEMs), with nanometer-scale control of ionized species and precise regulation of their complex three-dimensional (3D) topography [210]. When the deposition of self-assembled monolayers onto an underlying surface is dictated by a specific micropattern according to a PDMS master, it is common to speak of microcontact printing technique (μ CP). Kidambi and colleagues combined these two described methods to generate a composite suitable environment for the co-culture of HEPs and fibroblasts [210] (**Figure 15**). They utilized two synthetic ionic polymers, the sulfonated polystyrene (SPS) and the poly(diallyldimethylammonium chloride) (PDAC), to build multiple overlapped PEM films, identified as (PDAC/SPS)₁₀, on the top of a standard tissue culture polystyrene surfaces. A micropatterned structure of SPS was further added on the topmost of the PEM films by means of the microcontact printing technology in order to avoid the previous documented cytophobic effect of the PEM surfaces on the HEPs [259]. The presence of fibroblast allowed the HEPs to remain attached to the 3D micropatterned PEM framework for up to three weeks without the aid of supplemental adhesive proteins. HEP-specific functions (albumin and urea) were also preserved or improved in these conditions. With this study, the authors wanted to highlight the suitability of both layer-by-layer deposition and μ CP techniques in creating templates of patterned co-cultures of HEPs and fibroblasts, permitting the control of cell-surface interactions and the long-term maintenance of the hepatic functions.

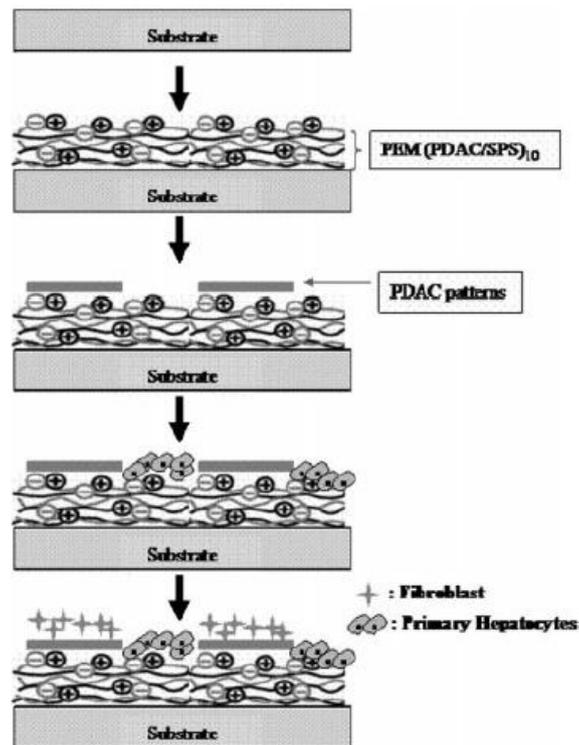


Figure 15. Scheme of the approach to pattern co-culture of HEPs and fibroblasts on PEM films [210].

Furthermore, these outcomes indirectly outlined the advantages of these techniques upon those formerly illustrated, such as the high fidelity, ease of duplication, ability to print a variety of molecules with nanometer resolution, and lack of harsh chemical treatments [210]. PEMs can be alternatively constructed with natural polymers. Kim et al. [218] selected chitosan and hyaluronic acid, respectively as cationic and anionic polyelectrolytes, to assemble PEMs in a 3D manner for the co-culture of HEPs and LSECs. By means of this 3D PEM architecture of chitosan, well-known compatible substrate for the culture of the HEPs, and hyaluronic acid, biomaterial used for the culture of endothelial cells, the authors wanted to recapitulate the charged environment of the Space of Disse of the liver. Alternate layers of chitosan and hyaluronic acid were directly assembled on monolayer of HEPs, lying on a collagen-coated substrate. Next, LSECs were added on the top of the PEM-coated HEP complex. In this configuration, HEPs and LSECs remained physically separated by the interposed nanoscale PEM (comprising five or fifteen layers with respective heights of 30nm and 50nm); however, intercellular signaling and heterotypic interactions could be potentially promoted thanks to the high degree of hydration of the PEM. The LSEC-PEM-HEP system, incorporating the minor number of layers, allowed the simultaneous maintenance of phenotype of both cell populations and the hepatocellular functions sustain (albumin and urea secretion, as well as CYP450 enzyme activity) over 12 days of culture. Is it possible to infer how the choice of the biomaterial and the 3D configuration contributed to these favorable outcomes? In this regards, the sandwich-collagen gel model proposed by Bale et al. for the co-culture of HEPs and LSECs [181] should be taken into account. This co-culture model may represent an appropriate system to compare to the LSEC-PEM-HEP one with the scope to get some insights. Among the distinct co-culture configurations proposed in the study, one was prepared by seeding the HEPs between two layers of collagen, followed by the inoculation of the LSECs on the topmost layer of collagen which, in turn, were covered with another collagen film (**Figure 16**). Such 3D cell organization, analogous to that of the

LSEC-PEM-HEP system, did not allow directly contacts between HEPs and LSECs. In addition, similarly to the previous investigation, the collagen was selected as suitable biomaterial to provide the cells with native cues, since it is one of the major components of the space of Disse. The decision to use a collagen gel sandwich derived from previous researches demonstrating its beneficial effect in the long-term maintenance of the HEPs. In such co-culture arrangement, the albumin production showed similar level as the control monoculture of only HEPs on day 12. This adverse result was ascribed to the presence of two layers of collagen gel on the top of the HEPs which may have negatively affected the metabolic functions of the hepatic cells and the loss of LSEC phenotype.

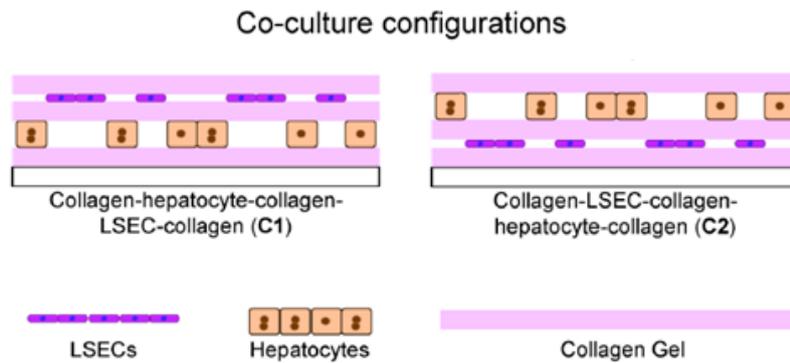


Figure 16. Two different sandwich-collagen gel configurations proposed by Bale and coworkers for the co-culture of hepatocytes and liver sinusoidal endothelial cells (LSECs). Image was modified from [181].

This outcome may induce us to assume that the choice of a biomaterial and its characteristics (as, for instance, the thickness) may principally impact on the hepatic activities and that, accordingly, chitosan-acid hyaluronic in the form of PEM better impact on the HEPs. However, this is not completely true. In fact, Bale et al., exploring other sandwich-collagen gel co-culture configurations, demonstrated that different cell localization in the system, namely inverted positioning of HEPs and LSECs (**Figure 16**), provoked a dramatic increase of albumin secretion after 12 culture days, significantly higher than that measured in the previous co-culture pattern, as well as the preservation of this activity over 30 days. In contrast with the preceding end, this finding proved that the biomaterial was not the main responsible of the optimal hepatic response. Rather, cellular culture and organization were likely implicated. Other mechanisms (discussed in the previous paragraph) may be involved and that may be vaguely attributable to the choice of the biomaterial. One major drawback of collagen sandwich culture is its failure in capturing heterotypic interactions [180]. Indeed, layer-by-layer design strategy shows shortcomings due to the solution of the soluble cell-adhesive electrolyte that is directly deposited on the cell monolayer and establishes binds with the oppositely charged electrolyte on the surface. This solution may be toxic for the cell type, thereby, care must be taken in this context [252].

❖ CELL SHEET TECHNOLOGY

A different strategy for constructing 3D patterned co-cultures with a higher degree of heterotypic interactions is based on switchable surfaces and takes advantage of the properties of thermally responsive polymers. This technique was first introduced by Yamato et al. in the early 2000s [260]. In principle, patterned cell sheet are created on thermally responsive polymers, detached by reducing the culture temperature below the lower critical solution temperature of the polymer and transferred on monolayer of a second cell

population. In this manner, the co-culture setting is constituted. The poly(N-isopropylacrylamide) (PIPAAM) is a broadly used thermally responsive polymer; it is hydrophobic at 37°C permitting cell adhesion and growth, but it rapidly hydrates and becomes hydrophilic at 32°C, facilitating the spontaneous detachment of intact cell sheets in which the cell homotypic interactions are preserved. Usually, the PIPAAm is grafted to the dish and then patterned by using a mask (for example, glass coverslips in case of preparation of square cell sheets). HEPs and endothelial cells (ECs) were efficiently co-cultured by using this cell sheet engineering technology exhibiting an optimal morphological preservation [179]. Later, Kim et al. [182] revised this technique offering an approach to create a more precise and uniform stratified dual layer culture system of HEPs and ECs (**Figure 17**). In this study, the detachment of a monolayer of ECs, cultured on temperature-responsive culture dish (TRCD), occurred by using a gelatin-coated manipulator and reducing the temperature so that the cell sheet could attach to the gelatin. The EC sheet was transferred to the HEP monolayer and detached from the manipulator by increasing the temperature. In this configuration and in presence of EC sheet, the HEP-specific functions (albumin and urea production) were improved in comparison with the case of HEP monoculture. This result may be due to the increased degree of cell interactions as outlined by the stable expression of tight junctions. Such methodology presents the potential advantage of developing co-cultures with even more than two cell types.

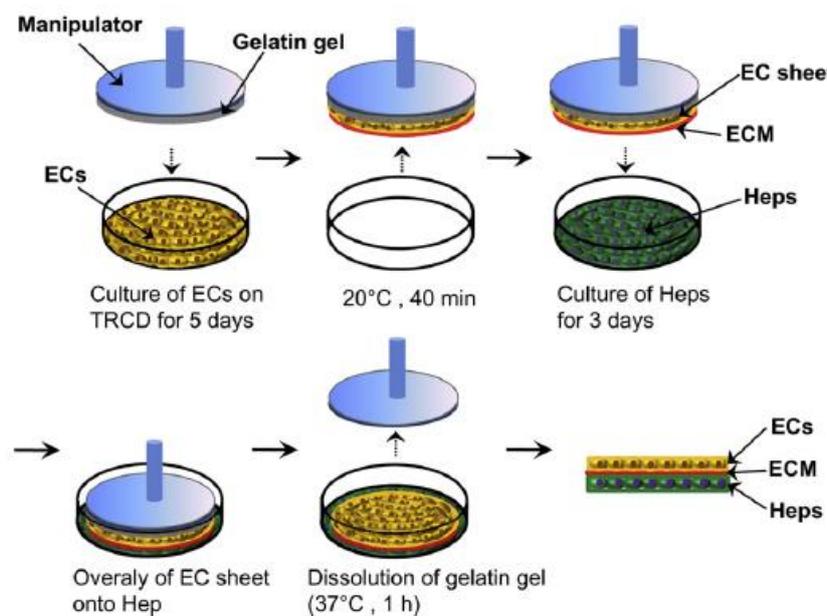


Figure 17. Technique for creating 3D patterned co-culture by using the switchable cell sheets principle [182]. Cells sheets, cultured on temperature-responsive culture dish (TRCD), are transferred on a monolayer of a second cell type by the usage of a gelatin-coated manipulator in order to setup the co-culture. Detachment and attachment of cell sheets is temperature-dependent.

In this regards, Cheng et al. [261] proposed feasible modifications of the technique. They employed photolithography to generate microheater array underneath a PIPAAm surface for the purpose of controlling the adhesion of specific cell types in defined patterned areas of the substrate. The regulation was made possible by turning on certain heaters under the polymer to increase the local temperature and switch small areas for favoring cell adhesion of one cell type at the time. In such a manner, subsequent cell types can be aligned to the first in accordance with the determined patterns permitting a precise special localization of the cell types. Shortcomings exists linked to temperature gradients around the heaters [252].

2.3.3.1.4. CELLULAR SELF-ASSEMBLY SPHEROIDS

With a different approach, investigators tried to exploit the apparent benefit of the heterotypic interactions in a maximized way for the maintenance of HEP functions *in vitro*. Already in 1952, Moscona and Moscona observed the ability of chondrogenic and myogenic cells of an early chick embryo to spontaneously aggregate together when co-cultured under suspension conditions [262]. Thus, later in 1961, they essayed to promote this cellular self-assembling process by using a rotational technique in order to generate interactions between different cell populations [263]. In 1989, Koide and coworkers published about the first discovery of the HEP spheroids [264]. A spheroid is defined as the final product of the self-aggregation of cells; it mimics natural physiological phenomena and aspects in an *in vitro* context. As stated by Lin and Chang [265], the formation of a spheroid is a three-step process that involves i) an initial rapid aggregation of dispersed cells by the establishment of cell-surface integrin binds, ii) a delay period for cadherin expression and accumulation, and iii) the final cell compaction in the form of the spheroids through homophilic cadherin-cadherin interactions. The spheroid is analogous to avascular tissue with diffusion limitation of about 150-200 μm to many molecules, especially O_2 [265]. Spheroids require culture conditions with efficient mass transport in order to avoid the accumulation of metabolic waste inside their body [265]. HEP spheroids exhibit phenotype differences than monolayer cultures, viz. higher cell viability and detoxification capability as well as re-establishment of their polarity [265]. A study demonstrated that primary porcine hepatocytes differently expressed more than 65 genes when underwent spheroid formation. This outcome led the authors to suppose that such transcriptional changes may be the cause of the close similarities of the spheroid features with the liver tissue [266].

The conjugation of co-culture facets and 3D spheroidal configuration acquired interest and popularity between several research teams with the end to include superiority of *in vivo*-like details in the *in vitro* hepatic substitute. Disparate strategies have been developed for the formation of single cell spheroids (generally called homo-spheroids), which were then adopted for the generation of multiple cell type spheroids (typically termed hetero-spheroids). These methods share a key aspect that is pivotal of the spheroid production; namely, facilitating the adhesion between cells to detriment of cell attachment to the culture environment. Based on the various culture parameters that promote mutual cell adhesion, the most commonly used spheroid-formation techniques can be broadly distinguished in two categories: chemical-based and physical-based techniques. The former includes those methodologies which exploit the chemical properties of selected materials to favor cell-cell adhesion and spheroid formation. Indeed, the second one comprises technologies which apply physics (for instance, precise motions or gravity force principle) for promoting the spheroid production.

❖ CHEMICAL-BASED METHODS

The generic approach here is to prevent cells to spread on a substrate and therefore favor their aggregation. In general, it can be outlined that hetero-spheroid formation is based first on HEP aggregation that may occur before or concomitantly to supporting cells addition. Numerous materials have been utilized for the formation of spheroids in an analogous co-culture manner.

In the resulting hetero-spheroidal configuration, HEPs generally exhibited higher expression of albumin and CYP450 mRNA, but the enhancement of effective functions (albumin release, urea secretion) is not always as significant, in comparison to the control of HEP homo-

spheroids. It is recognized that the establishment of tight homo- and hetero-interactions, may favorably impact HEP functionalities [200, 250].

Lu et al. demonstrated that galactosylated PVDF membranes can effectively support HEP adhesion and stimulate HEP spheroid formation through the specific interaction between galactose ligands on the substrate and asialoglycoprotein receptors on HEP surface [170] (**Figure 18**). After HEPs aggregation in spheroids, fibroblasts were added preferring to adhere to the HEP spheroids. The hetero-spheroidal configuration provided homo- and heterotypic contacts as well as cell-substrate interactions which may have contributed to the enhancement of the hepatic functionalities.

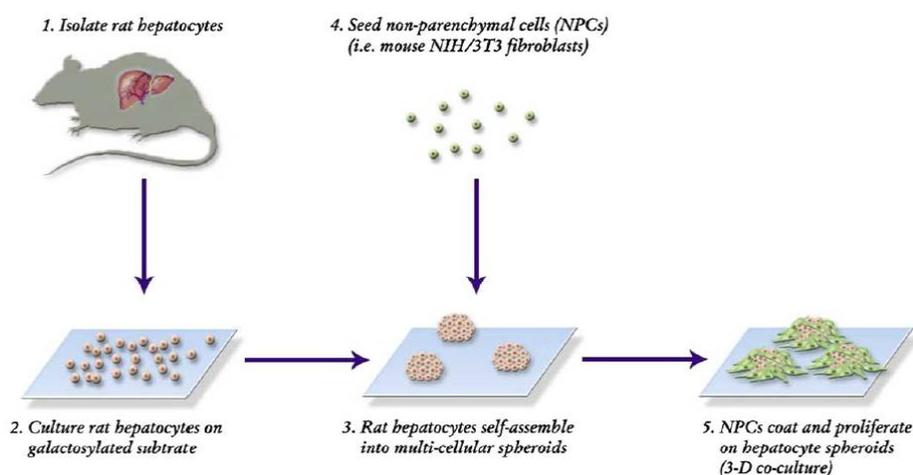


Figure 18. Schematization of the procedure of the hetero-spheroids formation between rat hepatocytes and fibroblasts [170].

On the other hand, Eudragit S100 polymer was used as a matrix. HEPs and the whole fraction of NPCs, cultured in presence of this polymer, agglomerated together from the first day of culture. Within four culture days, cells completed the spheroid formation. The hetero-spheroidal configuration provided homo- and heterotypic contacts as well as cell-substrate interactions which may have contributed to the enhancement of the hepatic functionalities. However, synthetic function (albumin production) was drastically increased, whereas biotransformation (CYP450) ability underwent a moderate augmentation.

In additional studies, spheroid formation between different cell populations was achieved by using several micropatterning techniques so as to establish a spatial control on the cells during the process. Fukuda et al. [207] realized a spheroid co-culture system by using soft-lithography, exactly micromolding technology. Photocrosslinkable chitosan polymer was patterned with a PDMS mold and crosslinked with ultra-violet light. The resulting chitosan microwells (200 μm in diameter and 50 μm deep) were used for the spheroid production. HEP cell lines were seeded as first, followed by the inoculation of the fibroblasts. Interestingly, cells preferred aggregation between themselves to adhesion onto chitosan. The authors justified this behavior with the high level of cell-cell adhesion molecules (cadherin and claudin) expressed by the cells and implicated in the spheroid formation. With a similar approach, the group of Lee generated micropatterned hetero-spheroids in PDMS-based concave microwells (300-500 μm in diameter). These latter, fabricated by soft-lithography, were coated with BSA in order to impede cell adhesion. In a first study [201], HEPs and HSCs were mixed together and simultaneously seeded into the microwells. Later, the investigators extended the complexity of their *in vitro* model by co-culturing HEPs, HSCs, and LSECs in accordance with the precedent protocol [198]. This technique provided spheroids highly uniform in size, which was dependent on the microwells diameter. Concave

microwells crucially controlled the cell aggregation into spheroids. In both studies, the hetero-spheroids generated an improvement of the hepatic functions (albumin production) which was more important when the three types were present. Such beneficial effect was maintained over 27 culture days. Despite the extreme control of the size and cell composition of the spheroids, a conceivable drawback of this method is the scant number of produced spheroids (one per well) that may be constraining in case of BAL application. Therefore, this technique might be more suitable for drug screening and predictive toxicology.

❖ PHYSICAL-BASED METHODS

Concerning the physical-based procedures, the production of spheroids relies on the retained capacity of the cells to re-organize in a tissue structure without the use of scaffold materials.

HANGING DROP METHOD

The hanging drop method takes advantage of the gravity to enforce the cells to self-assemble. In principle, this method, introduced by Kelm et al. [267], uses small aliquots (typically 20 μ l) of a cell suspension which are seeded on a tissue culture lid. This latter is subsequently inverted so that the aliquots of cell suspension turn into hanging drops that are kept in place due to surface tension [268]. In this manner, cells, under gravity effect, accumulate at the tip of the drop, at the liquid-air interface, and aggregate together. Some hanging drop plates have been commercially developed for the purpose of simplifying the cell seeding step, performed in appropriate wells, and the spheroid harvesting, thanks to the inclusion of a trap plate under the culture plate. The hanging drop technique is also based on cells natural tendency to adhere to each other [268]; likely, this justifies the fact that a single cell population has been prevalently utilized with this technique in order to avoid the difficulties associated with different adhesion degrees of the several cell types. However, Messner et al. [219] produced so-defined liver microtissues by employing HEPs, LSECs, and KCs in a hanging-drop culture platform (**Figure 19**).

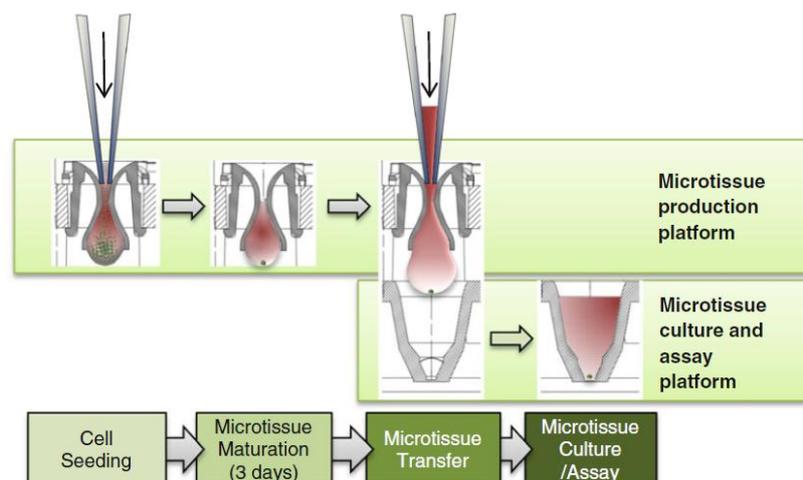


Figure 19. Hanging-drop culture technique for the production of liver microtissues made of HEPs, LSECs and KCs [219].

The resulting cellular construct resembled liver-like cell composition. HEPs exhibited prolonged lifetime and functionality in comparison with 2D culture, as well as long-term chronic and inflammation-mediated toxicity. The authors proposed the usage of the model

at the early time point in drug development. Similarly to the precedent illustrated methods, the simple and reproducible hanging drop technique, which enables the formation of size-controlled spheroids with a precise cell composition, allows the production of scant amount of spheroids which may be insufficient for the BAL application.

STIRRING TECHNIQUES

Concerning such demand, other approaches exploit the motion of suspended cells to obtain a massive spheroids production. The general principle is that a cellular suspension, placed into a container, is kept in movement so that cells do not adhere to the container walls, but instead form cell-cell interactions [268]. The movement has been established in different ways, for instance, by the aid of rotating and rocking systems as well as magnetic stirring in a spinner flask. These systems generally present a large size that, consequently, permits seeding of an important amount of cells enabling large yields of formed spheroids. Moreover, the motion of culture fluids is thought to assist the transport of nutrients to, and waste products from, the spheroids [268]. Here, the culture conditions (medium change, spheroid harvesting) are simpler than those of the precedent described techniques. Thomas et al. [197] sustained the production of hetero-spheroids of HEPs and HSCs by rotating motion. The spheroid formation occurred within two days following several distinct phases; HSCs first adhered and spread on the culture plate while the HEPs attached to the former cells, finally HSCs contracted and pulled the HEPs into focal aggregates which ended to form spheroids. Although the cells were not continuously maintained in suspension but got through an initial adhesion step, the resulting hetero-spheroids retained specific hepatic functions, including the CYP3A enzyme activity, which may be associated to the deposition of ECM components. According to the authors, HSCs, rather than the rotating technique, were crucially responsible of the promotion of spheroid formation, which inversely was slowed down in their absence, as well as of the hepatic functional enhancement, by means of the establishment of cell-cell interactions, release of soluble mediators and ECM production. Differently, Bao et al. [246] utilized a rocked mixing technique to create hetero-spheroids constituted of HEPs and adipose mesenchymal stromal cells. In this co-culture setting, rocking motion maximized the frequency of cell-to-cell contact and rapidly improves the rate and efficiency of spheroid formation. Therefore, spheroids produced rapidly and exhibited homogenous size as well as composition of the two cell populations. Functional activities (albumin and urea secretion) were highly supported in this co-culture system for 14 days. The authors also stressed the contribution of the mesenchymal stromal cells in forming the spheroids and stabilizing their integrity.

Ota et al. [222] proposed a technique for rapid formation of size-controlled hetero-spheroids consisting of HEPs and endothelial cells (ECs) by using micro-rotation flow. In principle, collagen-coated HEPs and ECs were collected in the center of a chamber by a micro-rotation flow. This chamber was composed of three layers with two inlet channels tangential to a cylinder in the middle layer and two outlet channels tangential to the cylinder in the upper layer (**Figure 20**); it represented the place where cells entered in contact and aggregated within two minutes. Cell aggregation was hydrodynamically controlled, by changing the cell density of the medium, resulting in the regulation of the spheroid size. In addition, the dispersion of ECs in the hetero-spheroids was uniform. Hepatic functions were not evaluated in this study.

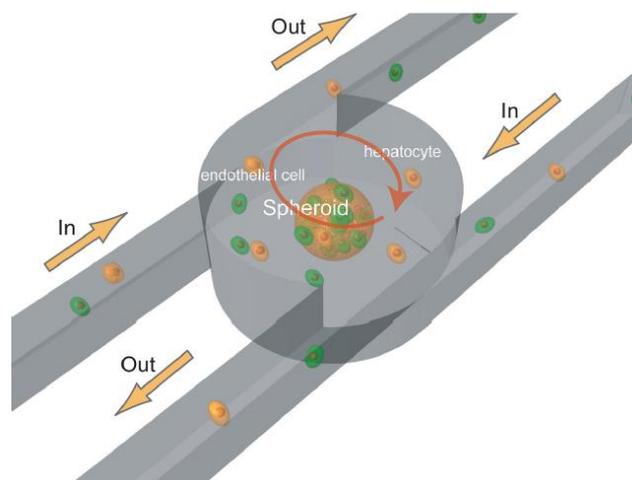


Figure 20. Method proposed by Ota et al. [222] for the rapid formation of hetero-spheroids, constituted of hepatocytes and endothelial cells, by the application of a micro-rotation flow. Spheroid production occurred in a chamber produced by soft lithography.

Spinner flasks were amply used for the production of homo-spheroids. Lee et al. [221] utilized siliconized spinner-flasks in order to prepare large quantities of hetero-spheroids constituted of HEPs and ECs for more specific BAL application. Hetero-spheroids formed 24 hours after seeding and exhibited an external layer of ECs covering the HEP spheroid. As stated in this study, such cell disposition may be a restoration of *in vivo* structures. However, ECs tightly adhered to the HEPs that may be due to their high attachment affinity for the HEPs. ECM production and cell-cell interactions were also observed in these co-culture conditions which both may play a role in the preservation of the hepatic functionalities. Similarly, Leite and colleagues [177] produced hetero-spheroids of HEPs and fibroblasts in spinner vessels. Spheroidal self-assembly of the two cell populations was observed shortly after cell inoculation. In these conditions, drug detoxification (evaluated by phase I and phase II enzyme activities) and synthetic (by measuring albumin secretion) capabilities of the HEPs could be maintained for up to 21 days.

Limiting aspect of these latter massive spheroid production methods is the lack of spheroid-size control that, depending on the application (as for example in the drug toxicology field), might require subsequent manual selection of similarly sized spheroids to setup comparable analysis or, even more dangerously, may lead the spheroids to acquire large diameters impeding the correct exchange of molecules and determining the appearance of a necrotic core.

2.3.3.1.5. MICROFLUIDIC DEVICES

Other studies [206, 223, 225], indeed, intended to maximize the level of preservation of the liver functions by reconstructing the 3D sinusoidal microenvironment. For this goal, microfluidic systems were developed. Interestingly, Yamada et al. [206] proposed an efficient method to produce complex micro-organoids that closely resembled the *in vivo* hepatic cord structure. Thus, a PDMS microchannel device was first fabricated via soft lithographic technology and then used for creating alginate hydrogel microfibers, incorporating HEPs and fibroblasts, according to a specific designed pattern. The alginate solution, entrapping the individually suspended cell populations, was introduced in the microchannel with a syringe pump (Figure 21 (a)) and crosslinked in hydrogel microfibers by means of a gelation solution. Crosslinking process contributed to pack the cells in the fiber core (Figure 21 (b)) resulting in the efficient formation of micro-organoids. With such

method, the authors not only realized homo- and heterotypic cell-cell interactions but also regulated the positioning of multiple cell types with micrometer-scale precision. Micro-organoids contributed to enhance HEPs viability, metabolic activity (albumin and urea secretion), biotransformation genetic expression, and differentiation. As reported in the study, this fiber-based cultivation process is advantageous because it enables high-density culture without significantly interrupting the flow inside the flow-through BAL device as well as a short diffusion length that is ideal for efficient plasma detoxification. Furthermore, the production of the cell-incorporating fibers is easy and performable in large amounts. Multiple microchannels may be thought to work in parallel in the same system.

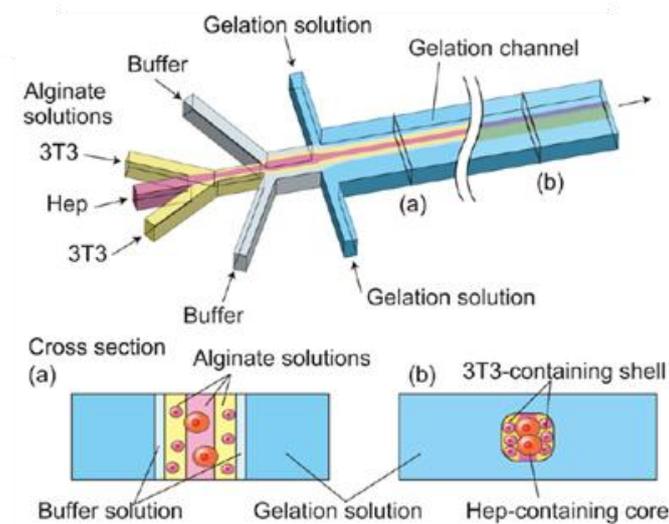


Figure 21. Microchannel device used by Yamada et al. to produce 3D alginate hydrogel microfibers, incorporating HEPs and fibroblasts, in order to mimic the *in vivo* hepatic cord structure of the liver. The fabrication of the cell-incorporating microfibers included (a) the introduction of the cell suspension in the microchannel and (b) the subsequent crosslinking of the alginate contributing to pack the cells in the microfibers.

2.3.3.2. WHICH OPTIMAL PARAMETERS DO DEFINE THE MOST PROMISING CO-CULTURE SETTING FOR AN ACTUAL CLINICAL BAL APPLICATION?

It is beyond dispute that co-culture beneficial affects the HEP viability and functionality *in vitro*. Nonetheless, it is still difficult to identify the optimal conditions that can provide the best co-culture setting to be transferred in the BAL application. However, preliminary design criteria can be delineated.

The analysis of the different co-culture approaches proposed in the literature seemed to reveal that the presence of only one assisting cell type can be sufficient to generate important improvements of the hepatic functions. This finding may result from an easier procedure for controlling and maintaining only one additional cell population *in vitro* in comparison to the presence of multiple supporting cell types. Among the myriad of non-parenchymal cell types co-cultured with the HEPs, the murine 3T3-J2 fibroblasts presented superiority in stabilizing HEP phenotype and supporting their functional behavior [249, 269]. Based on the importance of re-establishing a tissue-like situation to provide HEP with cues of the natural environment, the co-culture system should include more similar *in vivo* conditions. Fibroblasts do not give any; in the native liver, they are not in physical contact with the HEPs [205] and minimally collaborate with these latter. The literature lacks in studies that cautiously employ the main three sinusoidal lining cell types (LSECs, HSCs, and KCs) of the liver in the same system. As previously claimed [10], these cell populations

actively collaborate with the HEPs supporting their activities. Therefore, this domain still remains scantily or unexplored and may provide different outcomes than those available at present.

There is strong evidence that HEPs receive induction signals from the supporting non-parenchymal cell type in the co-culture system. According to Bhatia et al. [269], the propagation of this signal has limited distance. Therefore, culture width between the different cell types must be reduced. Diminution of the culture area is also in line with the design criteria of a bioreactor that must be optimized by balancing hepatic functions and surface area available for cell seeding [269]. Non-parenchymal cells occupy consistent surface area for mass transfer; therefore, the definition of an effective ratio may be necessary. Yet, this selection is extremely dependent on the cell population co-cultured with the HEPs. Additionally, an adequate capture of a signal may be accomplished by putting cells in contact. In this regards, the co-culture techniques which maximize the cell-cell interactions may be favored. Despite the open researches, these cell-cell interactions may also beneficially contribute to the maintenance of the HEPs functionalities. Furthermore, the choice of the methodology i) must take into account the necessity of a large quantity of co-culture constructs and ii) has to be associated with the type of BAL. For instance, hetero-spheroids are easy to scale-up compared to other constructs, as those obtained by micropatterning, and they can be generated in big amount (for example, by using spinner vessels). Hetero-spheroids may represent the optimal choice for the usage in bed bioreactors, because they can be easily fit in the alginate beads housed in the device. Anyway, certain other technologies cannot be taken into account for the realization of co-cultures for the BAL application since they do not meet the requirement described above and would require the setup of unpractical and inappropriate culture conditions (for instance, large surface area of functional HEPs for the definition of sandwich co-culture configuration).

Although much remains to be defined in this field and the real application of a co-cultured product seems to be far away from the present, the available outcomes are promising for continuing the works in this area.

3. RESEARCH APPROACH ESTABLISHED IN THIS THESIS

In accordance with the results found in literature and discussed above, the present research work tried to propose a novel approach of co-culture with the intent of fulfilling the gaps left from the previous studies. Here, the final goal was to define a co-culture construct which can be exploited in the fluidized bed bioreactor in future investigations. To reach the aim, several questions came to light, including the following:

1. Which and/or how many supporting cell types should be co-cultured with the HEPs?
2. Which ratio should be the cells co-cultured in?
3. Which technique should be the cells co-cultured with?

whose answers helped in the definition of the co-culture system.

Initial fundamental elements of this research approach (type and number of assisting cell populations) were selected in accordance with the physiological cell composition of the *in vivo* hepatic sinusoids. As already mentioned, these latter are constituted by four non-parenchymal cell types (NPCs) which actively collaborate with the HEPs in the performance of the liver functions. The idea, here, was to use the **three major liver-derived** sinusoidal non-parenchymal cells populations (i.e., **LSECs**, **KCs**, and **HSCs**) simultaneously with the HEPs in the same co-culture system. In this way, we intended to allow the cells to recreate certain conditions of the natural environment while providing some *in vivo*-like cues for the

preservation of this complex system in the long-term. Considering the shortage of human hepatic cells, the rat was selected as cell source in order to assure the availability of the cellular component for the definition of the co-culture construct. The cells were freshly isolated from the same rat liver and distinctly separated in three enriched fractions so that to have a better control of the cellular composition, at the seeding time, of the final co-culture product. Getting inspiration from the relative number of cells in the liver (60% HEPs and 40% NPCs), the isolated cellular fractions were cultured with **different ratios** for the purpose of establishing an optimal one able to provide the highest HEP functional performance. Under these conditions, the obtained cell populations were cultured according to a technique that could fulfill two fundamental criteria: i) maximization of the cell-cell interactions, and ii) massive production of the final co-culture construct. Therefore, the fresh primary hepatic cells were cultured under continuous orbital oscillation on anti-adhesive coated-dishes that promoted the cell self-aggregation in **hetero-spheroids**. This culture approach is schematized in **Figure 22**. The beneficial effect of the three NPC types in the hetero-spheroids was evaluated in terms of enhancement and preservation of several hepatic functions over 10 days of culture.

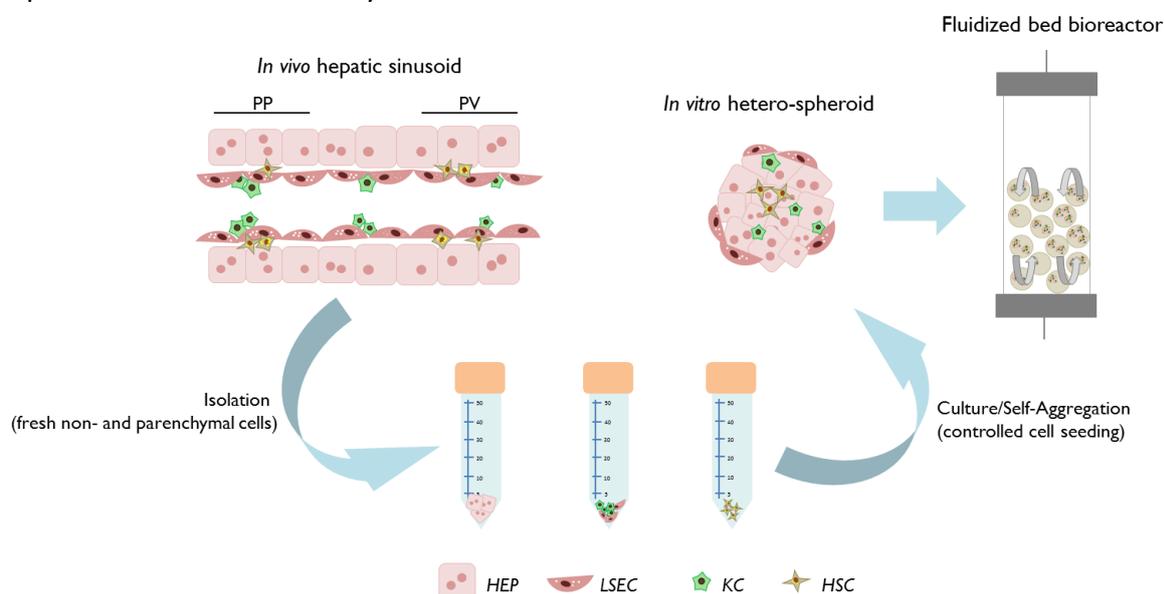


Figure 22. Schematic representation of the co-culture approach defined during this research work. Getting inspiration from the natural hepatic sinusoid, the present method co-cultured the three main sinusoidal non-parenchymal populations, namely the liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), and the hepatic stellate cells (HSC), together with the hepatocytes (HEP). These cells were freshly isolated, separated in three distinct fractions, and cultured according to controlled seeding in order to finally self-aggregate in the hetero-spheroids. These latter represent suitable models to be used in the fluidized bed bioreactor.

The hetero-spheroids represented the optimal construct to be employed in the fluidized bed bioreactor, designed in our laboratories. The bioreactor is the main component of the BAL device since it houses the cells. These latter are encapsulated in turn in alginate beads which protect cells from mechanical stress and immune-system component attack. Thus, the established hetero-spheroids were encapsulated in alginate beads and the cellular behavior was investigated. Preliminary studies in the (reduced scale) fluidized bed bioreactor were additionally carried out, but further analysis must be considered.

In line with the studies in the literature, our co-culture approach supported the HEP activities by taking advantage of the presence of multiple cell types, incorporated under controlled cell seeding conditions. Metabolic/synthetic functions were preferentially

sustained over those of biotransformation/detoxification. Such cell response may result from the production of soluble factors, likely secreted by KCs, which may have adversely affected on the phase I and phase II enzyme activities (as in the acute phase response of the liver). However, this co-culture platform may represent a promising tool to preserve the hepatic functionalities in the bioreactor while bridging acute failure patients to organ transplantation or liver recovery. Other cell types, of different origin, should be tested in such configuration in order to get insight on the efficacy of this co-culture product.

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CHAPTER 2

CELL ISOLATION AND CHARACTERIZATION

"Cell separation must, I feel, be viewed in the most general of terms where 'a population of cells is depleted of cells which share particular characteristics'." – P.T. Sharpe, 1988.

1. INTRODUCTION

The procedures of cell isolation have faraway beginnings. The first example was, likely, that of neuronal fibers isolated from frog by Harrison and colleagues in 1907. Nevertheless, the preparation of isolated cells from solid organs was for long time considered unachievable [1].

In the case of the liver, HEPs (HEP) seemed to be inseparable from its neighbors and, thereby, impossible to isolate. Initial attempts, employing mechanical action for the cell separation, yielded low damaged cells. The failure of these empirical methods was especially ascribed to the lack of knowledge of the properties of the components implicated in cellular adhesion [1]. With the evolution of laboratory technical facilities and biological understanding, progresses were done in the definition of cell separation methodologies. In 1967, Howard et al. reported the first successful isolation of intact HEPs from rat liver, although in low yield (3-5% of the original liver) [2]. The breakthrough of this isolation technique was the injection of a balance-saline solution containing enzymes (i.e. collagenase and hyaluronidase) into the major sinusoids of the rat liver. With this study, the authors gave evidence that intact HEPs could be isolated from the organ. Moreover, it became clear that enzymes had a role in the performance of the cell isolation and that a better exposure of the connective tissue of the liver to the enzymes could lead to a better survival of the HEPs [1]. Based on this concept, in 1969, Berry and Friend succeeded in isolating high yield of intact HEPs (30-50% of the original liver) by perfusing a rat liver with an oxygenated Ca^{2+} -free balance-saline solution containing collagenase and hyaluronidase [3]. During perfusion, the liver swelled remarkably and the collagen matrix was digested and loosened, allowing the release of the cells during a shaking step, following to perfusion. Such procedure was identified as 'one-step' method. Since then, many modifications of the original method or new isolation approaches have been proposed for the purpose of obtaining HEPs with better preserved characteristics. Important variations were introduced by Seglen (1972-1976) [4]. These proposed changes were based on studies demonstrating the pivotal role of Ca^{2+} in the procedure of HEPs isolation. In fact, it is well-know that these ions are essential for collagenase activation. Yet, considering the dependence of the function of cell adhesion molecules on the interaction with Ca^{2+} , the lack of these ions in the perfusates determines the loosening of gap and tight junctions, as well as desmosome cleavage, between HEPs. Thus, Seglen defined the so-called 'two-step' method, consisting in an initial perfusion of the liver with Ca^{2+} -free perfusate to allow the irreversible cleavage of the hepatic desmosomes, followed by the perfusion of a solution containing collagenase and Ca^{2+} , this latter added in physiological or suprphysiological concentrations [1, 5]. The Ca^{2+} -free solution could or not be supplemented with Ca^{2+} chelating agent, such as ethylenediaminetetraacetic acid (EDTA), that could favorably contribute to the desmosome cleavage by sequestering the ions to the hepatic junctions. However, as stated by Berry [5], the two-step method yielded identical results than the one-step method in terms of yield and time required for isolated cell preparation. Accordingly, the use of two solutions was considered to add an unnecessary degree of complexity to the procedure. The supplement of Ca^{2+} was thought to be worthless because the collagenase contains sufficient bound Ca^{2+} for full activity which, in addition, do not interfere with desmosomal cleavage [5]. Nevertheless, the two-step method of Seglen was amply used by different research groups in the following years including slight minor modifications at the protocol and it is the most used one at present. Moreover, this technique can be applied to the liver of various species [6].

Simultaneously, the isolation of liver sinusoidal non-parenchymal cells gained interest among the researchers, thus, separation protocols were sought. In such circumstances, the

possibility of obtaining not only HEPs but also non-parenchymal cells from the same cell suspension, resulting from the perfusion of the rat liver according to the one- or two-step technique, became evident. HEPs were/are easily separated with high purity from the rest by means of low-speed centrifugation steps; this is thanks to their very large size and density compared with the non-parenchymal cells (NPC). Whereas, the separation of three NPCs (liver sinusoidal endothelial cells – LSEC –, Kupffer cells – KC –, and hepatic stellate cells – HSC –), present in the supernatants at low concentration in a relatively large volume, posed difficulty due to the overlap of their buoyant densities. At the beginning, the NPC populations were not separated one by one from the initial cell suspension. Their overall recovery rather occurred after treating the hepatic cell suspension with pronase enzyme that selectively destroys the HEPs leaving the other cell types [7, 8]. However, once the resulting NPC fraction was seeded onto glass or plastic, only certain cell types (predominantly the KCs) were favorably maintained in culture over other populations that, indeed, disappeared [9]. Therefore, different methods were necessary. In 1976, Knook and Sleyster described a separation procedure using countercurrent centrifugation in an elutriation rotor which resulted in purified fractions of viable KCs and LSECs [10]. The isolated cells were only subject to a microscopic analysis whilst their functions were not evaluated; however, the use of pronase in the perfusion of the liver, prior to centrifugal elutriation, seemed to negatively impact on cell functionality and integrity [9]. Thus, Praaning-van Dalen and Knook presented a method for the isolation of KCs and LSECs that used pronase at low temperature in the perfusion of the rat liver in order to reduce damage of surface structures [11]. With a similar approach, Knook and Leeuw described a procedure for the isolation and purification of HSCs, employing initial enzymatic perfusion of the liver with pronase and a final purification step by centrifugal elutriation [12]. With the same purpose, other investigators utilized different substances (e.g. enterotoxins) in alternative to the pronase. A clear shortcoming, common to these techniques, was the impossibility to isolate several hepatic cell types from a single liver [9]. Another limiting aspect of these isolation methods was the involvement of specialized equipment, such as elutriation centrifuges, to which not all laboratories have access. Moreover, it has been claimed that centrifugal elutriation alone is not useful for selection of a specific cell types from the mixed NPC suspension considering the similar centrifugal densities of the constituting cells [13]. Thus, different strategies were proposed for the purpose of providing several undamaged cell types from the same liver by using basic laboratory facilities. The most widely used procedures utilized differential or density centrifugation steps in combination with gradients compounds (including Percoll, metrizamide, arabinogalactan, or Nycodenz). In 1985, Smedsrød et al. presented a simple and rapid technique for the isolation LSECs and KCs [14]. In this method, the cell suspension, obtained by perfusing the liver with collagenase, was first subject to low-speed centrifugation to pellet the HEPs; next, the saved supernatants were centrifuged on a two-step Percoll gradient which resulted in an accumulation of LSECs and KCs around the intermediate band. This mixed fraction of LSECs and KCs was further separated by taking advantage of the different adhesion behavior of the two cell populations for selective substrates. Namely, the whole fraction was shortly seeded onto plastic or glass so that only the KCs could tightly attach; then, the remaining unattached cells, especially constituted by LSECs, were transferred onto fibronectin-coated surfaces. In such a manner, KCs and LSECs were obtained in high yield and purity implying, in addition, the selection of only functional intact cells as all the others did not attach to the substrates and were washed away during medium change [9]. Similarly, Blomhoff et al. isolated HSCs from the initial NPC suspension by Percoll density gradient centrifugation, collecting a paltry amount of the selected population [15]. In alternative, gradient of arabinogalactan [16, 17] or of Nycodenz

[18, 19] were used to isolate HSCs from rat livers. However, one major drawback here was the difficulty of obtaining isolates without other NPC contaminations [20].

To improve the purity of a cell population of interest, alternative isolation methods have been also described. More commonly, flow cytometry and side scatter-activated cell sorting represent standard techniques for cell separation by utilizing characteristic antibodies or taking advantage of some cell fluorescent features. HSCs were isolated thanks to the fluorescence associated with their storage of vitamin A; however, this method was not completely reliable considering the variable store of the molecule in the cells. In alternative, the immunomagnetic separation technique exploited the concept of identifying and binding specific antigens of a desired cell type by means of peculiar antibodies or lectins attached to small magnetizable particles. Once a magnetic field is applied, the cells attached to the magnetic beads can be attracted to the magnet and can be separated from the unlabeled cells. Based on this strategy, beads coated with SE-1 monoclonal antibody, uniquely expressed by the rat LSECs, were employed to purify LSECs from the rest of NPC types [21]. In another study, magnetic beads coated with CD45 antibody were utilized to separate periportal, midlobular and centrilobular LSECs based on the *in vivo* different CD45 expression in LSECs across the hepatic lobule [22]. Indeed, KCs were magnetically separated from the other cells after their loading with irons through phagocytosis.

Despite the myriad of separation protocols, only few attempted to obtain simultaneously parenchymal and non-parenchymal cells from the same liver. Yet, in these cases, cell contamination was still important. Recently, Pfeiffer et al. proposed a new approach to isolate human HEPs, KCs, LECs, and HSCs from the same donor tissue [23]. The protocol combined different techniques mentioned above. That is, cell suspension, resulting from the two-step EDTA/collagenase perfusion of resected human liver, was first used to isolate HEPs by means of low-speed centrifugation steps, followed by a Percoll density gradient step in order to remove non-viable cells. NPC-containing supernatants were loaded on a two-layer Percoll density gradient and centrifuged; the resulting cell fraction present at the interface between the two Percoll layers was used to separate the different NPC populations. Namely, such fraction was shortly seeded on a culture plastic plate allowing only the KCs to adhere. The unattached cells were subject to immunomagnetic separation. LECs, attached to CD31-labelled magnetic beads, were attracted by a magnet while the remaining CD31 negative HSCs passed through the column to be collected at the bottom of it. Removing the magnet, the LECs were eluted and collected at the bottom of the column. Few months ago, Werner et al. [24] published an isolation protocol which, similarly to the precedent one, focused on obtaining human HEPs, KCs, LECs, and HSCs from the same donor tissue. This method employed the initial collagenase perfusion of the liver, followed by low-speed centrifugation step in order to get the HEPs, density gradient centrifugation and magnetic-activated cell sorting to separate the different NPC populations. Differently from the previous method, this technique involved a diverse density gradient medium (iodixanol) in place of Percoll for the separation of the three NPC populations. Here, the HSC fraction were found in the upper layer of the gradient column after centrifugation and soon seeded; whereas, the KC/LSEC fraction were identified in the second layer of higher density and used in magnetic-activated cell sorting (MACS) columns for further purification. CD14⁺ and CD146⁺ MicroBeads were used to selectively purify the KCs and LSECs, respectively. Both isolation procedures provided cells in high yield and purity, which were slightly improved in the second technique.

All reviewed isolation protocols incorporated a section for the characterization of the separated cell populations.

Here, the aim was to establish an inexpensive protocol for the isolation of HEPs, KCs, LSECs, and HSCs from the same rat liver. Considering the later application of these cells, the protocol was established in accordance with some criteria. First of all, the technique had to provide exploitable cells in suspension fashion. Next, although cell purity was not the major constraint, the cellular composition of each isolated population had to be defined in order to better control successive seeding of the cell fractions. Based on these requests, some isolation techniques were more apt than others and the characterization methods were consequently chosen. The isolation protocol is described below.

2. METHODS

2.1. CELL ISOLATION AND SEPARATION

Hepatic parenchymal and non-parenchymal cells were isolated from 7-month-old male Sprague-Dawley rats (Janvier Labs, France) weighting 200-300 g by using the two-step method of Seglen [4] with minor modifications. Sorting of the different cell populations was achieved by combining previous procedures [25, 26]. The entire procedure is schematically represented in **Figure 1**. In short, the procedure started by anesthetizing a rat with pentobarbital (100 μ L per 100 g body weight). Once the rat was deeply asleep (verified by the absence of reflexes), it was lied down on its back on the surgical table, its paws were attached to the table with surgical tape, and its abdomen was cleaned with 100% ethanol. The following steps were all performed under laminar hood in sterile conditions. The rat abdominal cavity was exposed by cutting the abdomen along its median line and lateral openings. Other organs (intestine and stomach) were gently moved on the left side of the rat in order to identify the inferior vena cava and the portal vein. A 21G needle, connected to the perfusates, was carefully inserted into the inferior vena cava and secured by using a surgical clamp. A first 37°C solution, containing EGTA and 1% bovine serum albumin (BSA), was perfused (10.5 mL/min) in retrograde direction through the liver. As soon as the liver swelled, the portal vein was cut and the superior vena cava was clamped. The liver was rapidly washed out of the blood and turned to a beige color. Perfusion was maintained for at least 10 min. At this point, a second 37°C solution, consisting of Ca^{2+} and collagenase supplemented with 1% BSA, was perfused through the liver and continued (for at least 20 min) till some clues of matrix digestion appeared (namely, appearance of “goose pimples”, loss of elasticity and compactness, acquisition of a soft consistency). Thus, the digested liver were carefully excised with sterile scissor and forceps and transferred in a sterile petri dish filled with cold Dulbecco’s Modified Eagle Medium (DMEM). The capsule of Gibsson was peeled off and the liver was gently shaken into the medium in order to permit the cell release. The resulting liver cell suspension was filtered through 70 μ m cell strainers and stored in 50 mL Falcon tubes. These latter were centrifuged three times at 50 g for 5 min at 4°C. Both pellet and supernatant were saved after each centrifugation. Pellet was suspended in DMEM and mixed with Easycoll separating solution (Biochrom AG – Merck Millipore) with a final density of 36%; this mixture was centrifuged at 168 g, 20 min, 4°C. Pellet was only saved and washed in DMEM by centrifugation at 100 g, 5 min, 4°C. Pellet containing mature HEPs was retained. The three major hepatic NPC populations were separated from initially saved supernatants. Briefly, supernatants were split in two parts, each of which was employed to isolate either HSCs or a fraction constituted of KCs and LSECs. To obtain HSCs, supernatants were firstly centrifuged at 50 g for 5 min and HEP-containing pellets were discarded. This step was repeated till no pellet was formed. Thus, supernatants were centrifuged at 200g for 10 min. The resulting pellets were collected, suspended in 10 ml of

DMEM and centrifuged at 200 g for 10 min. The final HSCs-containing pellet was saved. Indeed, KCs and LSECs were obtained from the other portion of supernatants. These latter were initially centrifuged at 1350 g for 10 min. Pellets were collected together and suspended in 40 ml of DMEM. Each 10 ml of this cell suspension was loaded on a distinct 50 ml Falcon tube containing density gradient Easycoll separating solution (25-50%); the prepared four 50 ml Falcon tubes were centrifuged at 1350 g for 30 min. Cells placed at the interface between the two density cushions were collected, suspended in DMEM and centrifuged at 1350 g for 10 min. Pellet, containing a mix of KCs and LSECs, was retained. All different isolated hepatic cell fractions were suspended in hepatic culture medium (HCMTM, Hepatocyte BulletKit, Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco – Life Technologies). Cell viability was assessed by blue trypan exclusion test.

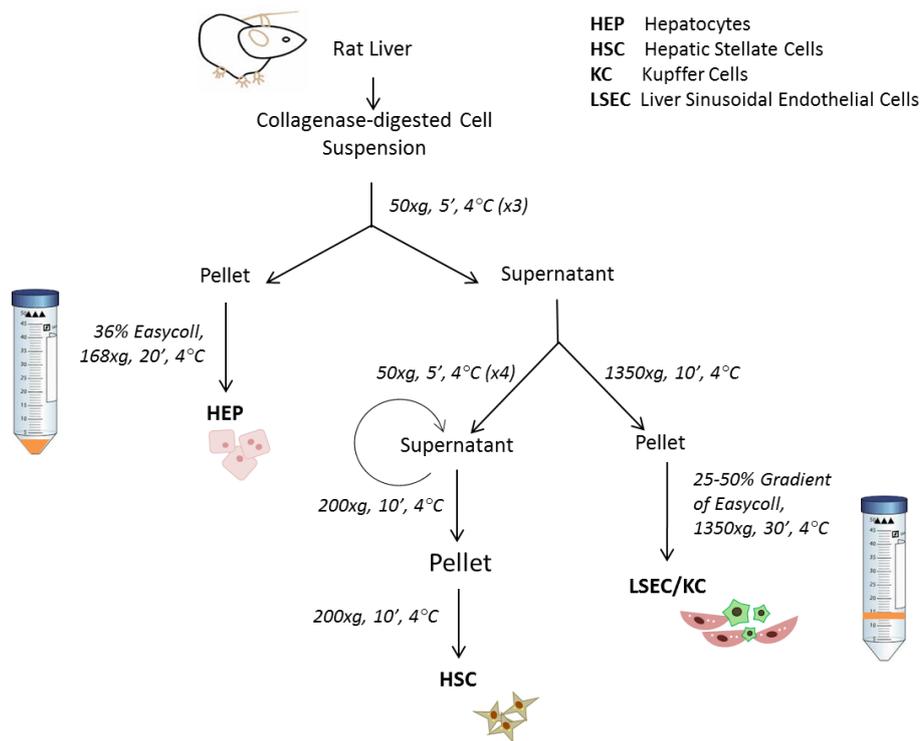


Figure 7. Schematic representation of the separation technique of parenchymal (HEP) and non-parenchymal cells (HSC, LSEC, KC) from a rat liver.

2.2. IMMUNOCYTOCHEMISTRY

Each isolated cell fraction was seeded in duplicate in separated wells of an 8-wells Lab-Tek culture chamber. Specifically, HEP-fractions was seeded on collagen-coated wells, KCs/LSECs fraction on fibronectin-coated wells, whereas HSCs on uncoated wells. Cells were incubated overnight at 37°C and 5% CO₂, in a humidified environment. Attached cells were washed out of unattached ones in warm phosphate buffered saline (PBS, pH 7.4, Gibco – Life Technologies), fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature (RT), and permeabilized in 0.5% Triton X-100 in PBS (10 min, RT). After blockage of unspecific sites with 3% (w/v) BSA solution (30 min, RT), cells were incubated with primary antibodies (1:150) for 1h at RT. Excess of primary antibodies was washed out and secondary antibodies (1:500) were added for 1h at RT in the dark. Cells were finally incubated with 4',6-diamidino-2-phenylindole (DAPI) for 1 min at RT in the dark and mounted in Prolong Gold Antifade with DAPI (Invitrogen). Images were acquired under epifluorescence microscope (Leica DMI 6000B, 20x/0.40). Used primary and secondary antibodies are listed in **Table 1**.

2.3. HSC PROLIFERATION

Cells constituting the HSC-fraction were stained with Ki67 antibody (Abcam) on day 1 and 3 following the procedure described in the previous paragraph.

2.4. ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY (ESEM)

The isolated cell fractions were individually seeded onto circular cover glasses (16 mm ϕ), previously sterilized, placed in single wells of a 12-wells culture plate, and coated or not with extracellular components, as reported in the previous paragraph. Cell seeding was carried out as described above. After overnight adhesion, cells were washed twice in PBS and fixed in Rembaum solution (at 2.5% glutaraldehyde). Samples were amply washed in distilled water and prepared for observation. Each circular cover glass was taken out from the culture plate, mounted on specimen stub, and coated with gold. The stubs were introduced in the stub holder ready to be observed under environmental scanning electron microscope (FEI Quanta 250 FEG) at 15.00 KV of voltage, 4 Torr of pressure, and 5°C in temperature.

2.5. FLOW CYTOMETRY

Each isolated cell fraction, kept in suspension, was first fixed in a Fixation Buffer (Biolegend) under gentle agitation for 20 min at RT in the dark, washed twice in PBS, and permeabilized into diluted (1x) Permeabilization Wash Buffer (Biolegend) under gentle agitation for 10 min at RT in the dark. Cells were incubated with specific diluted primary antibodies (1:20) for 20 min at RT in the dark, washed in 5 mM EDTA in PBS and incubated with secondary antibodies for 20 min. Cells were washed and kept in EDTA solution. All washing steps were performed by centrifuging the samples at distinctive speeds (HEP: 50 g, KC/LSEC: 900 g, HSC: 600 g). A minimum of 150,000 cells, suspended in a minimum of 150 μ l of solution, was considered for each condition. Analysis was performed on a Gallios™ flow cytometer (Beckman Coulter, Inc). Acquisition of >5,000 events were collected using 20mW diode blue laser and 525/40 (detected dye Alexa Fluor 488) and 575/30 (detected dye PE) bandpass filters, and 20mW diode red laser and 660/20 bandpass filter (detected dye APC). Used primary and secondary antibodies are listed in **Table 1**.

Table 1. Primary and secondary antibodies used to characterize isolated hepatic cells by immunocytochemistry (ICC) and flow cytometry (FC).

	Primary Antibodies		Secondary Antibodies	
	ICC	FC	ICC	FC
HEPs	Cytokeratin 18 ⁽¹⁾	Cytokeratin 18 ⁽¹⁾	Alexa Fluor 488 ⁽⁵⁾	PE ⁽⁶⁾
HSCs	Desmin ⁽¹⁾	Desmin (conjugated) ⁽²⁾	Alexa Fluor 594 ⁽⁵⁾	Alexa Fluor 488
KCs	CD68 ⁽³⁾	CD68 ⁽³⁾	Alexa Fluor 488 ⁽⁵⁾	PE ⁽⁶⁾
LSECs	SE-1 ⁽⁴⁾	SE-1 ⁽⁴⁾	Alexa Fluor 488 ⁽⁵⁾	APC ⁽⁶⁾

Antibodies were purchased from: ⁽¹⁾ Santa Cruz Biotechnology, ⁽²⁾ Abcam, ⁽³⁾ AbD Serotec, ⁽⁴⁾ Novus Biologicals, ⁽⁵⁾ Life technologies, and ⁽⁶⁾ BioLegend.

Commercial cryopreserved cells⁶ were used as positive controls for initial definition of the protocol.

2.6. IMAGE ANALYSIS

Immunocytochemical images were opened with ImageJ software and the nuclei of non- and positive-stained cells were counted. The count was performed on four different pictures, corresponding to four random areas of the seeded well, acquired from distinct cell isolations. Results were presented as mean \pm SD of the percentages of the stained cells upon the un-stained ones and used as estimation of the cell purity of each cell fraction.

3. RESULTS AND DISCUSSION

3.1. GENERAL ASPECTS

The entire procedure, starting from the step of rat anesthetization till that of cell seeding, lasted about 6-7 hours (approximately 1 hour for the phase of cell isolation from the liver and 5 hours for the separation of the different cell populations). In these conditions, minor changes were introduced to the original protocol in order to maximally reduce the cell stress/damage before seeding. First of all, the perfusion solutions were supplemented with BSA to protect the cells from collagenase attack and, in addition, all stages were performed at 4°C in order to reduce the cell metabolic activity. For the same reason, each obtained cell fraction was stored at 4°C while waiting for the seeding. Nonetheless, this long lasting may have negatively affected on HEP viability.

The proposed protocol represented a reproducible tool to obtain enriched fractions of the four main hepatic cell populations (parenchymal and non-parenchymal) simultaneously from the same rat liver. To wit, three fractions were finally obtained: one enriched in HEPs, another constituted of HSCs, and a last one formed by a mix of LSECs and KCs. The further separation of this latter cell portion into the two constituents was beyond the scope of this protocol; in fact, we rather defined the proportion between the two cell populations by characterization techniques. Yield and viability of each fraction, evaluated before cell seeding by trypan blue exclusion test, are referred to the total amount of cells isolated from rats with an average weight of 250 g; these parameters are reported in **Table 2**. Purity of each fraction is also reported in **Table 2** and was determined after immunocytochemical staining for cell-specific antigen as explained in paragraph 2.5. Data are presented as means \pm SD.

Table 2. Yield, viability, and purity of each isolated cell population. Data are referred to independent cell isolations from a 250 g average weight rat.

	HEP-fraction	LSEC/KC-fraction	HSC-fraction
Yield	79.66x10 ⁶ \pm 12.33x10 ⁶	14.91x10 ⁶ \pm 3.31x10 ⁶	5.97x10 ⁶ \pm 2.63x10 ⁶
Viability (%)	74.83 \pm 1.47	85.24 \pm 3.14	87.34 \pm 2.01
Purity (%)	96.0 \pm 1.56	LSEC: 53.38 \pm 15.57 KC: 31.80 \pm 2.23	--

⁶ Hepatic cells were isolated from adult Sprague-Dawley rats. Details of the purchases: HEPs (ref. RTCS10, Life Technologies), HSCs (ref. RA-6242, CellBiologics), KCs (ref. RTKCCS, Life Technologies), and LSECs (ref. RA-6017, CellBiologics).

3.2. HEP-FRACTION

HEPs were easily isolated by means of low-speed centrifugation steps thanks to their large size and density (1.10-1.14 g/ml [27]). Yield of this fraction amounted to $79.66 \times 10^6 \pm 12.33 \times 10^6$ per 250 g average weight rat, whereas the viability was $74.83 \pm 1.47\%$ (n=6) (Table 2). HEP viability was not as high as that of the other cell types probably because they are more easily vulnerable to damage, therefore, isolation procedure as well as long lasting before seeding may have negatively affected the cell viability. HEPs exhibited characteristic polygonal shape with an average diameter of $19.97 \pm 3.51 \mu\text{m}$, cytosolic vesicles and mono- or binucleation feature after overnight adhesion on collagen pre-coated wells (Figure 2).

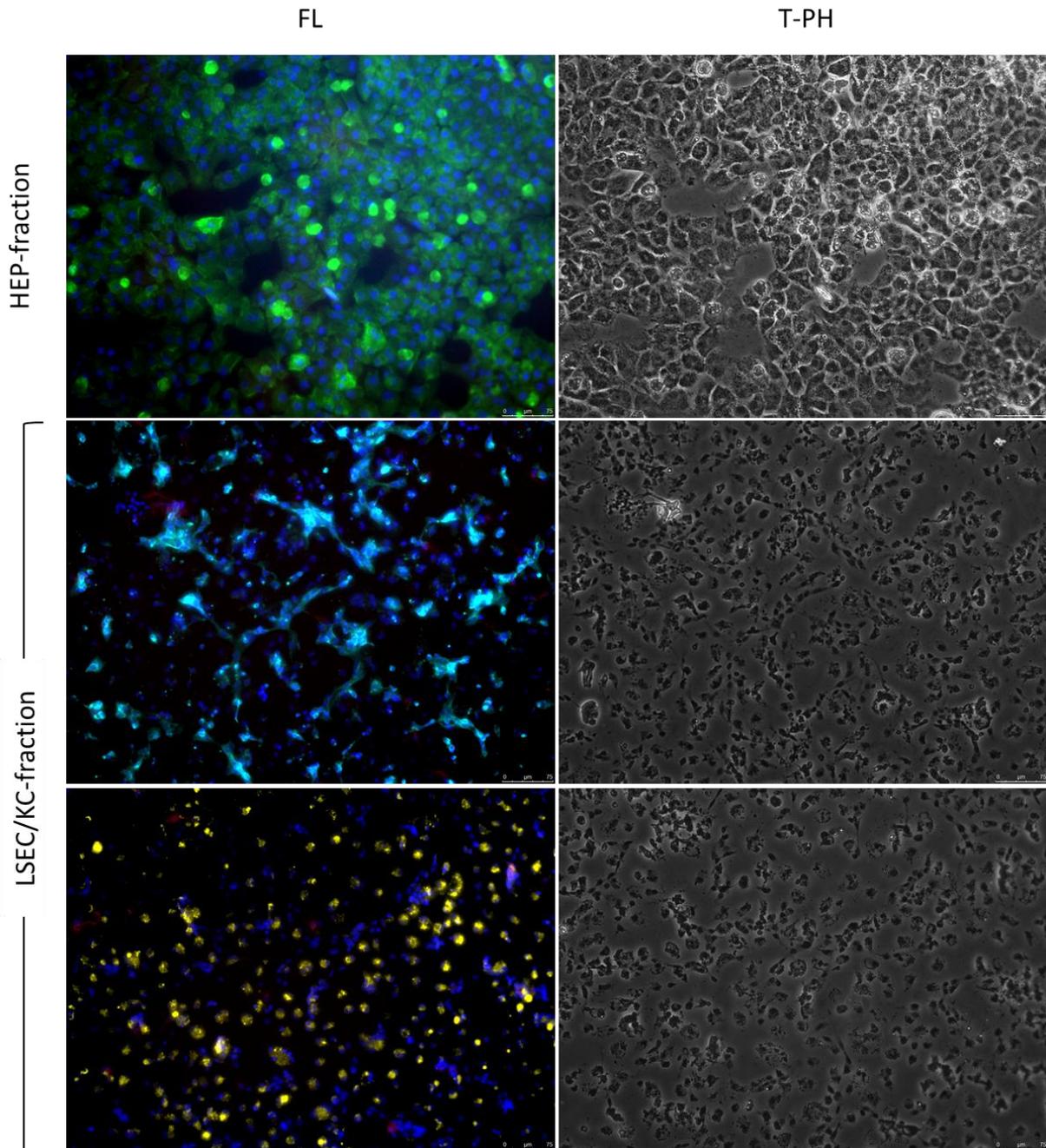


Figure 2. Immunocytochemical characterization of HEP-fraction and LSEC/KC-fraction. Fluorescence (FL) images of stained cells (HEPs in green, LSECs in cyan, KCs in yellow, and HSCs in red) were snapped under epifluorescence microscope (magnitude 20x) 24 hours after cell seeding. Additional phase-contrast images (T-PH) were included in order to better point out morphological features.

HEPs were positive for cytokeratin-18 marker (**Figure 2**) demonstrating a high purity of this cell fraction (>95%) as estimated by negative staining for the other used markers via immunocytochemical staining (n=4).

3.3. LSEC/KC-FRACTION

KCs and LSECs were obtained as cell mixture in a sole fraction. This latter was isolated from the same supernatant portion by centrifugation on a two-layer gradient solution of Easycoll (25 and 50% in density) and saving the cell suspension placed between the two density cushions. This fraction yielded to $14.91 \times 10^6 \pm 3.31 \times 10^6$ per 250 g average weight rat and showed a viability of $85.24 \pm 3.14\%$ (n=7) (Table 2). Because of an overlap of densities of these two different cell types (1.060 g/ml for KCs and 1.80 g/ml for LSECs [27, 28]), gradient centrifugation was not sufficient to provide purified populations of either KCs or LSECs. Yet, this step of the protocol was mainly designed to clear the final LSEC/KC-fraction from debris, dead cells and erythrocytes as well as hepatocytes and any other non-parenchymal cell type [27]. Accordingly, the isolated cell fraction resulted highly purified in terms of both KCs and LSECs as confirmed by specific immunostaining for CD68 and SE-1 (gold standard markers of KCs and rat LSECs respectively) indicating that the LSEC/KC-fraction was constituted of $53.38 \pm 15.57\%$ of LSEC and $31.80 \pm 2.23\%$ of KC (**Table 2**) (n=4). These values closely approach the cellular proportions of the *in vivo* organ [29].

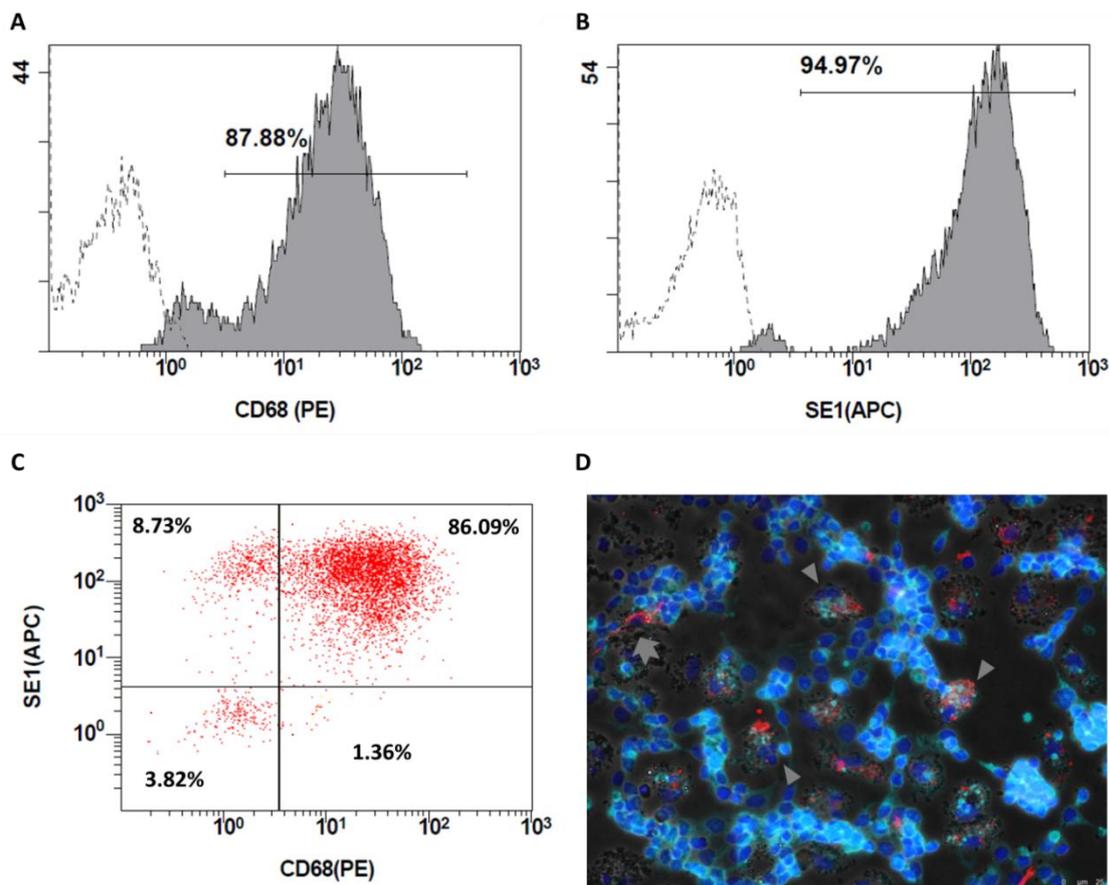


Figure 3. Flow cytometry analysis of the LSEC/KC-fraction. Double-staining of this fraction with specific antibodies for KCs (CD68) and LSECs (SE-1) revealed a high enrichment and purity of both cell populations as indicated in the histograms (**A – B**, dotted curves indicated unlabelled samples used as a controls and grey curves showed the positive-staining cells). A large portion of cells resulted stained with both antibodies (**C**) which may be due to a misleading uptake of both used antibodies from KCs (**D**, black arrows without tails).

A scarce immunoreactivity for desmin antibody ($3.52 \pm 2.41\%$) was also detected corresponding to a contamination of HSCs. Such purity results were further reinforced by flow cytometry (FC) analysis (**Figure 3**). Namely, the fraction resulted highly enriched with LSECs and KCs (with average values of $84.13 \pm 13.60\%$ for KCs and $89.91 \pm 6.35\%$ for LSECs) when double-stained with SE-1 and CD68 antibodies ($n=3$). Moreover, slight cell contaminations of HSCs ($0.67 \pm 0.70\%$) and HEPs ($5.17 \pm 2.57\%$) were also measured ($n=3$). However, the double-staining (SE-1+CD68) of the LSEC/KC-fraction led to an unexpected outcome; that is, an elevated percentage of cells stained by both used antibodies (Figure 3.C). This phenomenon may be arisen from a misleading uptake of both used antibodies from KCs as was also observed in immunocytochemical images, in which KCs presented a weaker fluorescence signal for SE-1 antibody (Figure 3.D, black arrows without tails).

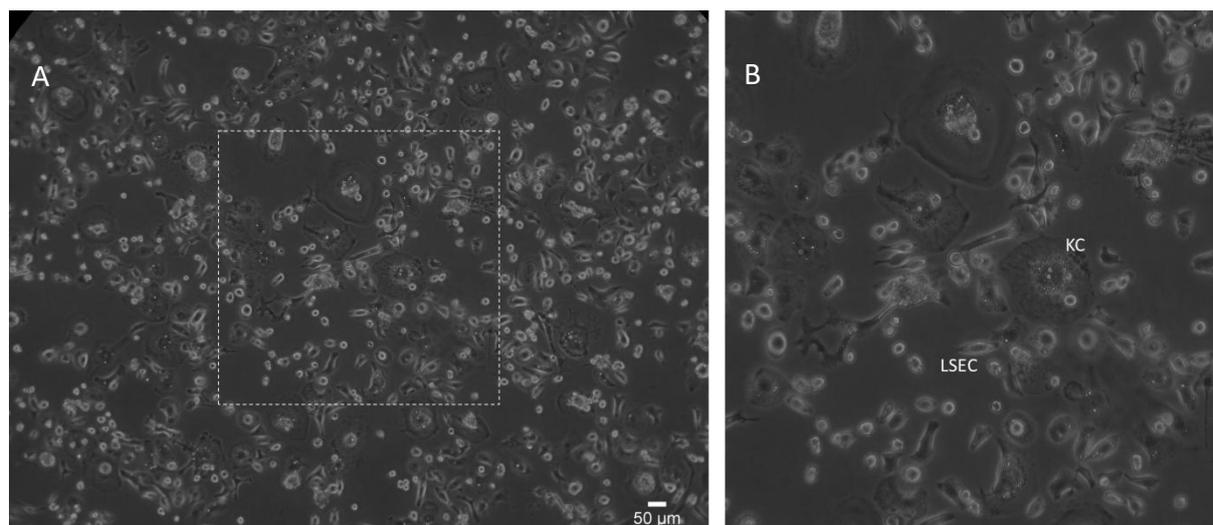


Figure 4. Morphological features of KCs and LSECs observed under optical light microscope (10x). (A) KCs and LSECs attached on fibronectin-coated culture plate after overnight incubation. (B) KCs and LSECs were clearly distinguishable by peculiar characteristics. KCs exhibited fried-egg shape, prominent nucleus, and abundant cytoplasm constituting of lysosomes and pinocytotic vesicles; whereas, LSECs showed spindle-shaped appearance with oval nucleus.

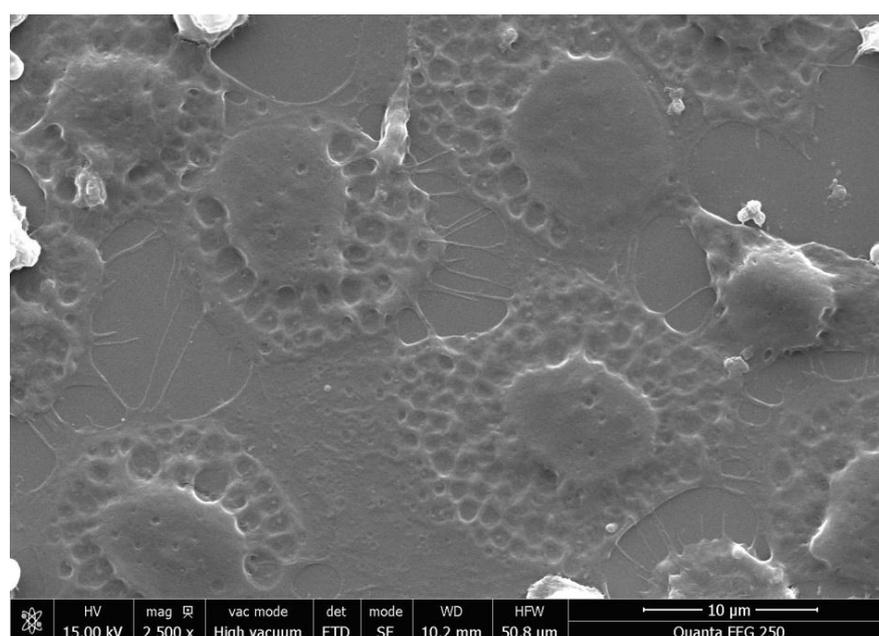


Figure 5. ESEM observation of LSEC fenestrae after overnight adhesion on fibronectin-coated culture dish.

Alternatively, this outcome may be due to the presence of small cell packets constituted of both KCs and LSECs which may be erroneously counted as only one event from the FC device. KCs and LSECs were also identified for some peculiar morphological features after overnight adhesion on fibronectin-coated wells. The former exhibited fried-egg shape, prominent nucleus with irregular shape and abundant cytoplasm constituting of lysosomes and pinocytotic vesicles whose content was variably translucent (**Figure 4**). LSECs, indeed, showed a spindle-shaped appearance (**Figure 4**) with an oval nucleus and tended to organize themselves in cell clusters (**Figure 3.D**). In addition, LSECs presented typical fenestrations (**Figure 5**) when observed under ESEM. Fenestrae are generally considered to be a reliable marker of LSECs [27].

3.4. HSC-FRACTION

HSCs are very low density cells (1.053 g/ml) associated with their small sizes and lipid droplets content [28]. Moreover, HSCs represent only a scant cellular component of the liver (6% of the total relative cell number [29]). These elements may have made difficult HSC isolation throughout the years. Here, HSC-fraction was obtained from a portion of supernatants by means of initial repeated low speed centrifugation steps in order to wash residual HEPs out. After last higher speed centrifugation, the HSC-containing pellet was partially resuspended so that to minimize the contamination of erythrocytes. This fraction yielded to $5.97 \times 10^6 \pm 2.63 \times 10^6$ per 250 g average weight rat and showed a viability of $87.34 \pm 2.01\%$ (**Table 2**) (n=7). It was not possible to estimate cell purity of this fraction because only a scarce amount of HSCs were positive-stained for desmin (gold standard markers of rat HSCs) after either overnight or 24 hours of culture; yet, they could be recognize under phase-contrast microscopy (cells indicated by black arrows in **Figure 6**). Such fact may be due to a poor spreading of the HSCs on the culture plate (in case of immunocytochemical case) as well as their round shape in suspension (for use in FC analysis). Otherwise, it may be associated to the absence of desmin in those freshly isolated HSCs with poor vitamin A content [20]. However, HSCs were present and exhibited typical star-shaped branches and oval nuclei (**Figure 6**). They also possessed lipid-rich droplets in their cytoplasm and especially located in perinuclear zones when observed under phase contrast (pinpointed by black arrows in **Figure 6**). These features disappeared by prolonging cultivation time; after seven days of culture, in fact, HSCs likely underwent activation process acquiring a myofibroblast phenotype that was recognizable from their shape change, enlargement of their nuclei, loss of lipid droplets, and proliferative facet (**Figure 7**). Both immunocytochemical and FC analysis revealed contamination by KCs (positive staining for CD68) of the HSC-fraction. Early studies have ascribed this inevitable issue of cell contamination to the similar densities of these two cell types and to their attitude to easily adhere to each other [28]. However, KC contamination seemed to decrease when HSC-fraction was cultured.

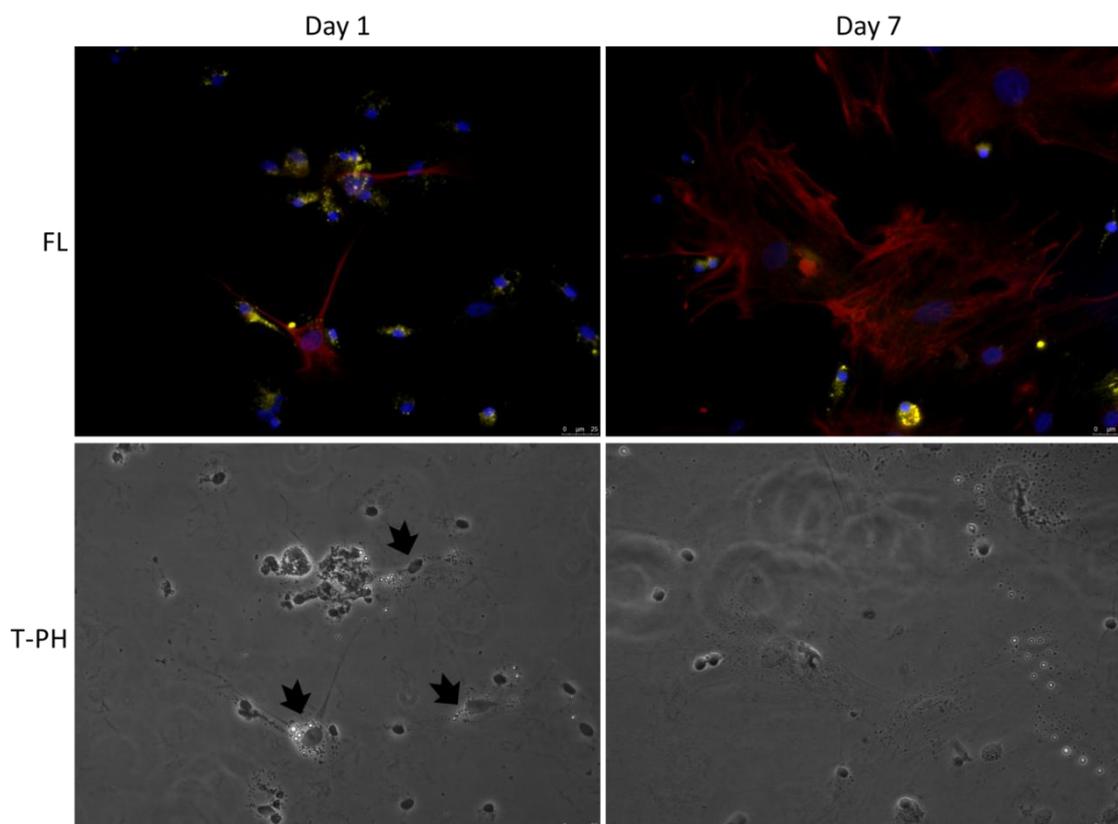


Figure 6. Immunocytochemical characterization of the HSC-fraction. Fluorescence (FL) images of stained cells (HSCs in red, and KCs in yellow) were snapped under epifluorescence microscope (magnitude 40x) 24 hours and 7 days after cell seeding. Phase-contrast images (T-PH) pointed out the presence of lipid-rich droplets in the cytoplasm of the HSCs which were especially located in perinuclear zones.

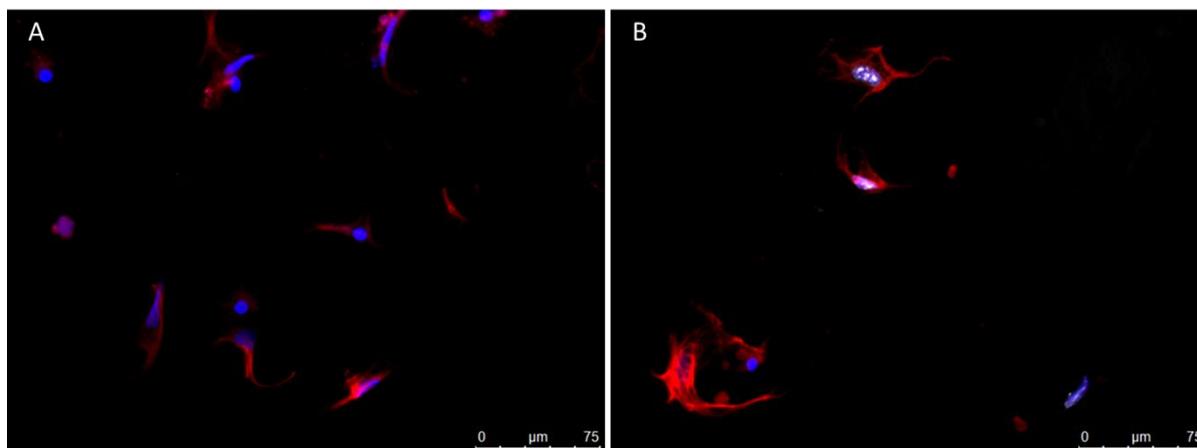


Figure 7. Proliferation of the HSC-fraction. Fluorescence images were snapped under epifluorescence microscope (magnitude 40x) 24 hours (A) and 3 days (B) after cell seeding. Ki67 antigen (grey) was detected on the protein situated in the HSC nuclei (blu). HSCs were distinguished by desmin positive-staining (red).

4. CONCLUSION

The proposed protocol for the simultaneous isolation and separation of HEPs and three major hepatic sinusoidal NPC populations was primarily defined in view of the final usage of the obtained cells. To wit, the concept was to utilize the four cell types in the establishment

of an *in vitro* liver co-culture model that required mixture and culture of the cells in suspension manner. Therefore, fundamental prerequisite was to get insight of the cell composition of each obtained cell fraction in order to better control the cell seeding in the definition of the co-culture model.

This procedure, based on previous similar techniques, represented a reproducible tool to obtain enriched fractions without involving the usage of complex equipment. In each specific separated fraction, corresponding cell types were clearly identified by means of peculiar morphological characteristics and immunofluorescence staining of cell type-specific antigens. Flow cytometry analyses were additionally performed to confirm purity outcome. In general, the technique provided a high enrichment of each fraction with the expected cell populations showing good purity. However, the protocol should be refined if it is thought to exploit it for other different applications. Moreover, the HSC-fraction should be better clear out of KC contamination.

The presented methodology may be adapted, with necessary modifications and improvements, for the isolation and separation of hepatic (parenchymal and non-parenchymal) cells from human liver resections.

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CHAPTER 3
BIOENGINEERING AN *IN VITRO*
SPHEROIDAL MULTI-CULTURE MODEL:
OPTIMAL RATIO FOR HEPATOCYTES/NON-PARENCHYMAL CELLS,
AND MEDIUM COMPOSITION

"It has become clear that hepatocytes in culture should be maintained in contact with either ECM or homo- or heterotypic cells to retain specific functions." – Marcus K.H. Auth, 1992.

ABSTRACT

The substantial demand of *in vitro* biologically active hepatocytes for drug-screening tests and therapeutic treatments (e.g. bioartificial liver devices) entails the definition of *in vivo*-like systems that can provide these cells with more physiological cues. In this work, we describe a certain number of culture elements that are essential for bioengineering an *in vitro* model that more closely resembles the liver situation, thus making it possible to better preserve the functions specific to hepatocytes and their viability. The model design consisted of assembling primary rat hepatocytes and major non-parenchymal sinusoidal cell populations (liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells) in a three-dimensional spheroidal configuration using a precise cell ratio within a controlled medium culture milieu. In order to establish the optimal conditions, several cell ratios and culture medium compositions were scrutinized by evaluating their combined effect on the albumin and urea secretion of hepatocytes over ten culture days. Our data suggested that the presence of non-parenchymal cell types beneficially impacted hepatocyte ability to produce albumin over time. This effect was non-parenchymal cell density-dependent; therefore, higher cell density implied greater protein secretion over time. This multi-culture impact nevertheless decreased when the culture was done in a highly supplemented medium. In this condition, the hepatocytes, whether or not in the presence of non-parenchymal populations, retained an almost equivalent capability for releasing albumin and urea. This may lead us to hypothesize that the inclusion of multiple non-hepatocyte cell populations and a highly supplemented medium may be crucial for the regulation of additional hepatic functions in the long-term. Although much still needs to be clarified, this spheroidal multi-culture model may be a suitable tool for different *in vitro* liver platforms. It retains the potential for providing more adequate responses to a variety of stimuli, thanks to the inclusion of non-parenchymal cells, because it maintains the functions of the hepatocytes. More functional hepatocytes may, in addition, result in a lesser need for these cells (such as, for instance, in bioartificial liver devices) and more reliable development of certain therapeutic treatments.

1. INTRODUCTION

The liver is a vital and complex organ which carries out a variety of functions indispensable for the correct performance of both other organs' duties, and the maintenance of human homeostasis. It is composed of an assortment of cell types [1], organized in a three-dimensional (3D) configuration according to specific relative proportions. Hepatocytes (HEPs) constitute 60% of the total cell number [2] and are the main functional actors in the liver, performing most physiological hepatic activities either alone or in collaboration with other hepatic cells [3, 4]. Paramount cooperative aid is provided by three non-parenchymal cell (NPC) types that inhabit the hepatic sinusoids, namely, liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs). LSECs form the sinusoidal wall and make up 20% of the total number of cells in the liver [2]. KCs are hepatic macrophages, constituting 15% of the entire cell population [2]. HSCs are the pericytes of the sinusoids, and represent 6% of hepatic cells [2]. These cell populations sustain HEPs via indirect interaction through release of mediators (e.g. cytokines and growth factors), or direct establishment of membrane contacts affecting hepatic functions in *in vivo* physiological and pathological circumstances [2, 5].

Defining functional *in vitro* engineered liver systems, that can be exploited in the construction of bioartificial liver (BAL) devices or drug toxicological screening models, should

take into consideration and mimic the complexity of the organ in order to overcome the limitations caused by *in vitro* phenotypical instability, loss of functional activities, and the short-term viability of HEPs. Some of the intricate *in vivo* facets, such as the 3D cellular arrangement and the presence of multiple cell populations, should thus be included in the *in vitro* culture setting.

On the one hand, spheroidal culture of HEPs may give the cells a suitable 3D environment, thus recreating physiological phenomena and characteristics [6]. In this confined configuration, cell-cell interactions are maximized and the HEPs survive longer, manifest extended liver-specific functions, and have a cyto-architecture similar to the *in vivo* cellular configuration [6-15].

On the other hand, co-cultures of HEPs with other assisting cells have been shown to be a real breakthrough, stabilizing and significantly improving hepatocellular functionalities over time, in comparison with single HEP cultures. These positive effects have been ascribed to the simultaneous manifestation of multiple events, reminiscent of actual *in vivo* occurrences [16, 17], that is, the establishment of homo- and heterotypic interactions as well as the secretion of soluble signaling molecules or insoluble extracellular matrix (ECM) components. Yet most co-culture studies, using diverse primary cell types or cell lines derived or not from the liver in disparate culture configurations, did not mirror the complexity of the liver's cellular composition, and included only one assisting cell population [16, 18-22]. As stated by Kostadinova et al. [23], however, a co-culture system lacking various hepatic NPC populations cannot summarize all the major functions of the liver. Recent models co-cultured two or three feeder cell populations together with the HEPs by controlling their seeding densities [24-28]. Prodanov et al. [28], for instance, presented a co-culture system showing facets of human liver physiology and functions, even though the cell selection was directed at cell lines derived or not from the liver. In addition to primary human hepatocytes, it included three different human cell lines (umbilical vein cells, hepatic stellate cells, and leukemic monocyte lymphoma **cells**) as substitutes for hepatic NPC populations. Furthermore, the actual cellular proportions between the different populations of the liver were overlooked when establishing *in vitro* systems. Still, determining an optimal co-culture ratio between HEPs and assisting cell type(s) is an important challenge for the design of a suitable model. Cells co-cultured in diverse ratios arrange themselves differently in the space of the system and, consequently, form interactions with different degrees of complexity that affect the cells' ability to release soluble factors, synthesize ECM matrix, and, thereby, sustain HEP functionalities. Setting-up *in vitro* engineered liver systems also requires the establishment of other culture parameters. HEP culture is challenging and depends on a suitable selection of the medium for short- or long-term preservation of their characteristics [29]. In addition, the composition of the culture medium appears to primarily impact the sustainment of hepatic functions [30]. The presence of multiple cell types in the same environment complicates the situation, as a single medium must be enough for all populations to avoid losing their phenotypes. Therefore, correctly defining the results of culture medium composition is critical for obtaining suitable cellular responses.

The scope of this study was to bioengineer an *in vitro* spheroidal multi-cultured liver model in which HEP viability and functions can be maintained over time. This was achieved by combining the culture parameters mentioned above, that is, 3D culture through a spheroidal approach, and co-culture of multiple hepatic NPC types with the HEPs isolated from the same liver according to a well-delineated ratio. LSECs, KCs, and HSCs were selected as ideal non-parenchymal supporting cells to be cultured with the HEPs as spheroids. Spheroids were cultured in different media formulated with diverse supplements which were strategically chosen to deduce the NPC-influence level on the preservation of HEP functionalities. With

this approach, we intend to answer two crucial questions concerning what optimal cell ratio and medium composition makes it possible to preserve both the viability and functionality of HEPs.

2. MATERIALS AND METHODS

2.1. HEPATIC CELL ISOLATION

Hepatic parenchymal and non-parenchymal cells were isolated from male Sprague-Dawley rats (Janvier Labs, France) weighing 200-300 g using the two-step method of Seglen [31]. Sorting the different cell populations was performed by combining previous procedures [32, 33]. Briefly, the resulting digested liver cell suspension was centrifuged (50 g, 5 min, three times). The resulting pellets were mixed and suspended in an Easycoll separating solution (Biochrom AG – Merck Millipore) prepared in Dulbecco’s Modified Eagle Medium (DMEM, Gibco – Life Technologies) with a final density of 36 %, and centrifuged (168 g, 20 min). The derived HEP-containing pellet was washed in DMEM (100 g, 5 min) and retained. The supernatants saved initially were split into two portions to isolate either HSCs or a fraction composed of KCs and LSECs. To obtain HSCs, the supernatants were first washed (50 g, 5 min), in order to discard the HEP-containing pellets. HEP-free supernatants were centrifuged twice (200 g, 10 min) and the resulting HSC-containing pellets were retained. The other initial supernatant portion was centrifuged (1350 g, 10 min) and the resulting pellets were mixed and loaded on a density gradient Easycoll separating solution (25-50 %), then centrifuged (1350 g, 30 min). Only cells at the interface between the two density cushions were retained, suspended in DMEM and centrifuged (1350 g, 10 min). The final pellets contained KCs and LSECs (termed LSEC/KC-fraction). The entire procedure was carried out at 4°C. Cell viability was assessed with the trypan-blue exclusion test on all the isolated cell fractions.

2.2. ESTABLISHING THE CULTURE CONDITIONS

Isolated hepatic fractions were suspended in 5ml of one of the four culture media – listed below –and supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco – Life Technologies). Suspended cells were inoculated into glass Petri dishes (ø x h = 60 x 20 mm) coated with Sigmacote® (Sigma-Aldrich). The multi-culture conditions included HEPs and the different isolated NPC fractions, in constant proportions of 20 % HSC-fraction and 80 % LSEC/KC-fraction. The multi-culture conditions were established in conformity with four distinct ratios (defined in **Table 1**). A mono-culture condition, constituted only of HEPs, was used as the control. Multi- and mono-culture conditions included 1×10^6 HEPs per dish. Cells were cultured under continuous orbital agitation at 70 rpm with an oscillation amplitude of 16 mm (SSL1 orbital shaker, Stuart) in a humidified environment at 37°C and 5 % CO₂.

Table 1. Culture ratios used for defining the multi-culture conditions.

CULTURE RATIO (HEP:NPC)	SEEDING CELL DENSITIES ($\times 10^6$)
1:0.5	1 HEP, 0.4 LSEC/KC, 0.1 HSC
1:0.67	1 HEP, 0.53 LSEC/KC, 0.13 HSC
1:1	1 HEP, 0.8 LSEC/KC, 0.2 HSC
1:2	1 HEP, 1.6 LSEC/KC, 0.4 HSC

Four days after seeding, the medium was switched with one that was serum-free. The culture was maintained for 10 days, during which the medium was changed every 3 days. Hetero-spheroids formed from the multi-culture condition, whereas homo-spheroids from the mono-culture one. All medium components were purchased from Sigma-Aldrich (France) unless specified otherwise. The four culture media used and their formulations are listed in **Table 2**.

Table 2. Culture media used and their compositions.

	MEDIUM COMPOSITION
WE+L-Glu+P/S	William's E medium without phenol red and L-glutamine (PAN BIOTECH) supplemented with 1 % (v/v) L-glutamine (PAN BIOTECH), and 1 % (v/v) penicillin/streptomycin (Gibco, Life Technology)
hmM	William's E medium without phenol red and L-glutamine (PAN BIOTECH) supplemented with 20 mM HEPES, 30 mg/l L-proline, 10^{-7} M dexamethasone, 1mM ascorbic acid (Sigma-Aldrich, France), 25 mM NaHCO_3 , 0.1 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 pM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 $\mu\text{g/l}$ epidermal growth factor (EGF), 10ml/l insulin-transferrin-selenous acid (ITS TM +Premix Universal Culture Supplement, BD Biosciences), and penicillin/streptomycin (Gibco, Life Technology)
HCM	Hepatocyte Basal Media (HBM TM , Lonza) supplemented with Hepatocyte BulletKit (Lonza)
HCM+ECGS	Hepatocyte Basal Media (HBM TM , Lonza) supplemented with Hepatocyte BulletKit (Lonza) and Endothelial Cell Growth Supplement (ECGS, ScienCell Research Laboratories)

2.3. MORPHOMETRY OF THE SPHEROIDS

Size evolution of the spheroids was followed by measuring their diameter (100 spheroids counted per condition) on images acquired under a light microscope equipped with a digital camera at defined culture time-points (days 4, 7 and 10). Image J Software was used for size measurement. Morphological details were also derived from these pictures.

2.4. ASSESSMENT OF THE VIABILITY OF THE SPHEROIDS

At day 10, the spheroids (about 20 μl) were incubated in a solution of propidium iodide (10 $\mu\text{g/ml}$) and of Hoechst 33342 (10 $\mu\text{g/ml}$) in Williams' medium E for 30 min at 37°C under gentle orbital oscillation. The spheroids were abundantly washed in Williams' medium and observed. Images were taken under epifluorescence microscopy (Leica DMI 6000B, 20x/0.40).

2.5. FUNCTIONAL EVALUATION

The 12-hour spent medium was collected on days 4, 7, and 10 from all conditions, and separately stored at -20°C. Albumin synthesis was quantified by ELISA test (Bethyl Laboratories, Inc.). Urea secretion was determined by colorimetric assay (BioAssay Systems). Tests were carried out according to manufacturer's instructions.

2.6. STATISTICAL ANALYSIS

For each independent experiment (n=3), results were presented as mean \pm SD. Statistical significance was determined using one-way ANOVA with Kruskal-Wallis test using the Prism - GraphPad tool. P-values <0.01 were considered to be significant.

3. RESULTS

3.1. HETERO- AND HOMO-SPHEROIDS: SIZE AND VIABILITY

Orbital agitating culture of fresh isolated rat HEPs with or without fractions of hepatic NPCs (HSCs and LSECs/KCs) led to massive production of cell aggregates of spheroidal shape, respectively named hetero- and homo-spheroids. Independently from the culture conditions (i.e. different co-culture ratio and medium), spheroid formation occurred within a few days (3-4). The time maintenance for spheroid integrity was subordinate to the type of culture medium used. In contrast, single culture of only NPC fractions (HSCs+LSECs/KCs) did not lead to any spheroid formation. Instead, the cells attached to the Sigmacote® culture plates (data not shown). Outcomes are presented in reference to the various media used.

WE+L-Glu+P/S medium

After four days of culture, hetero- and homo-spheroids exhibited heterogeneous sizes, ranging from 58 to 311 μ m. However, all spheroid categories presented a similar mean diameter that stabilized around 135 μ m (**Figure 1.B**).

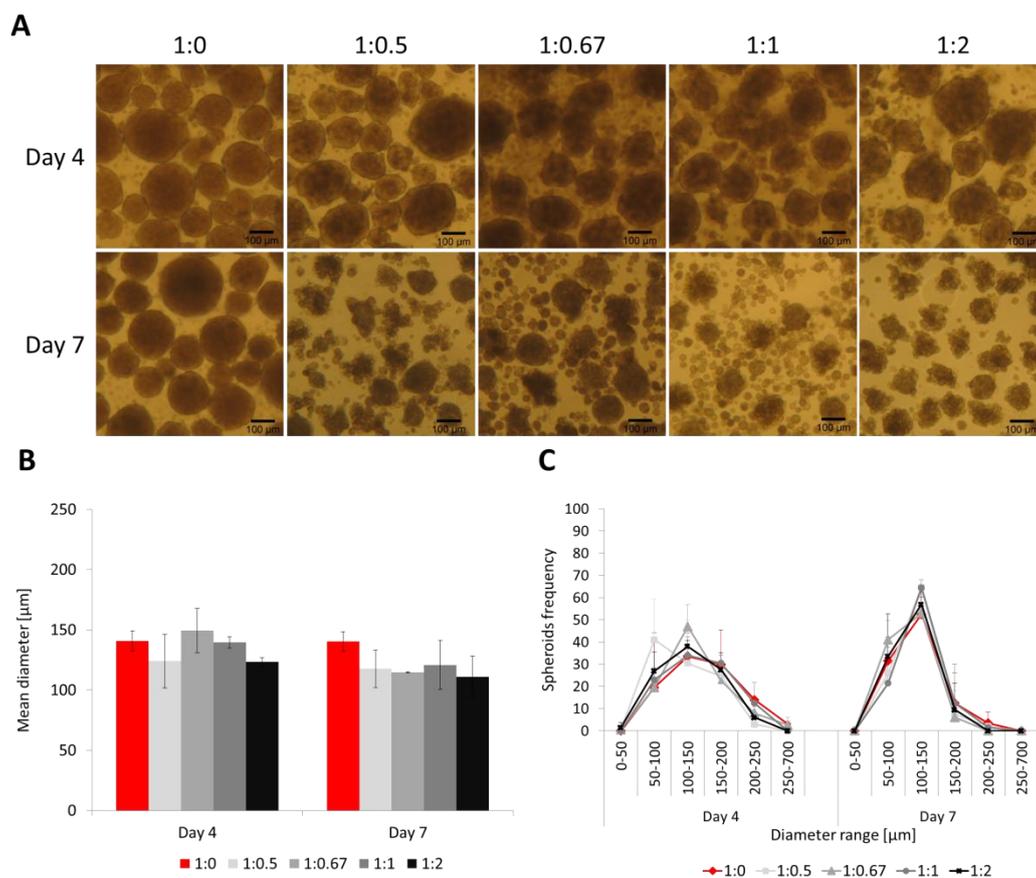


Figure 1. Morphometric evolution of homo- and hetero-spheroids in WE+L-Glu+P/S medium. **(A)** Changes in spheroid size were observed under an inverted light microscope at defined time-points (days 4 and 7). **(B)** Spheroid diameters were measured on the resulting images and used to evaluate the mean value for those obtained from independent experiments (n=3). **(C)** Spheroid frequency was evaluated in different size ranges by counting 100 spheroids per condition.

Hetero-spheroids from the 1:0.67 ratio had a larger mean size ($149 \pm 18 \mu\text{m}$) and, consequently, a higher number of spheroids measuring approximately $150 \mu\text{m}$ (**Figure 1.C**). While those from the 1:0.5 and 1:2 conditions manifested a smaller mean diameter ($124 \pm 22 \mu\text{m}$ and $123 \pm 5 \mu\text{m}$, respectively), the former displayed a higher quantity of small hetero-spheroids (**Figure 1.C**). The sizes were nevertheless not significantly different among the different culture conditions. On day 7, only homo-spheroids (1:0) preserved their integrity, exhibiting a round shape (**Figure 1.A**) with a mean diameter ($140 \pm 8 \mu\text{m}$) constant to the measurement on day 4 ($141 \pm 8 \mu\text{m}$). In all the other co-culture conditions, hetero-spheroids seemed to break apart so that single cells or small aggregates could be identified on the spheroid body or in suspension. As a consequence, the hetero-spheroid size reduced. The spheroids in the 1:0.67 ratio underwent the greatest size decrease ($115 \pm 0.5 \mu\text{m}$) (**Figure 1.B**). Consequently, we assumed that cell viability had decreased critically.

hmM medium

Homo- and hetero-spheroids showed different aggregation tendencies.

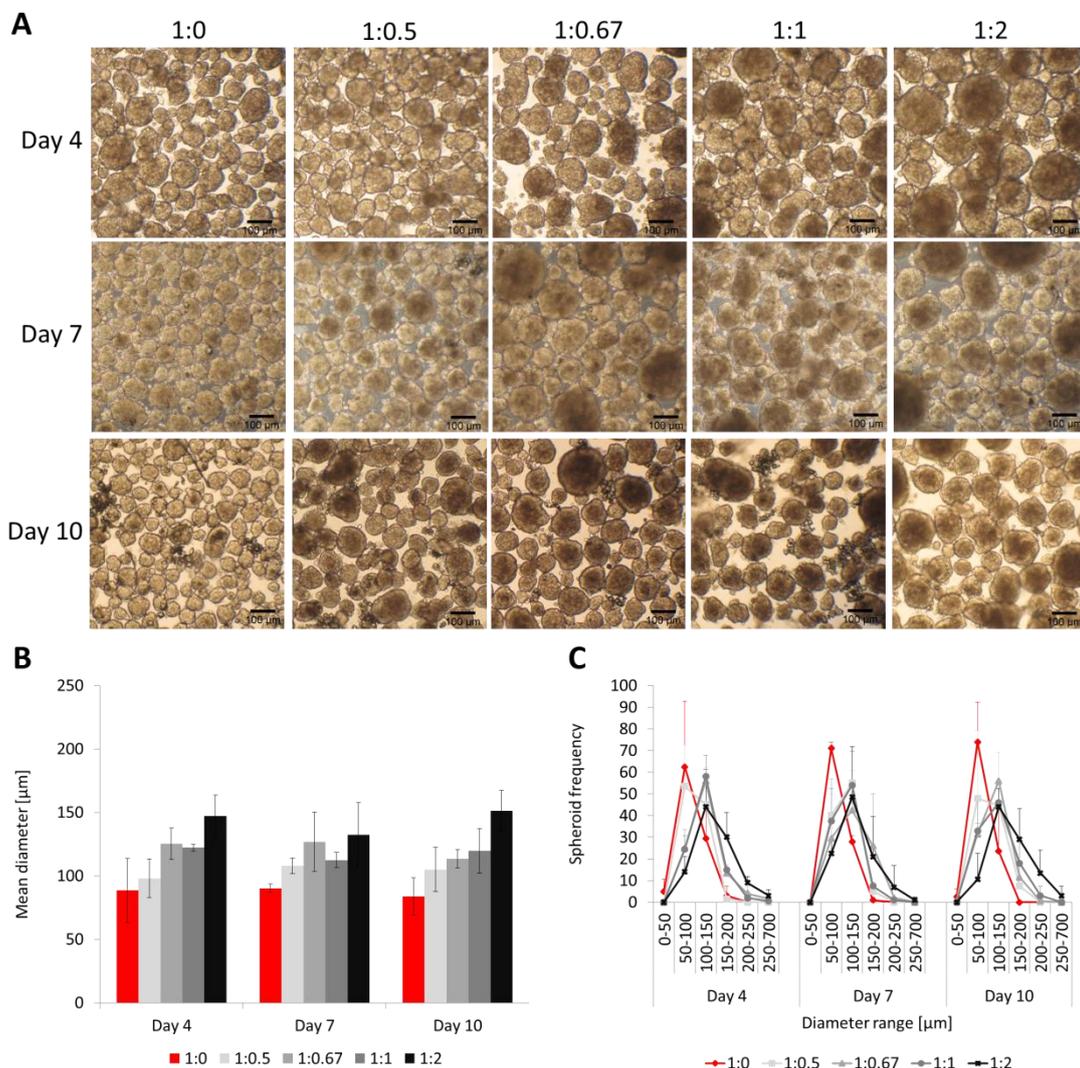


Figure 2. Morphometric evolution of homo- and hetero-spheroids in hmM medium. **(A)** Changes in spheroid size were observed under an inverted light microscope at settled time-points (days 4, 7, and 10). **(B)** Spheroid diameters were measured on the resulting images and used to evaluate the mean value for those obtained from independent experiments ($n=3$). **(C)** Spheroid frequency was evaluated in different size ranges by counting 100 spheroids per condition. Scale bar equals $100 \mu\text{m}$.

On day 4, homo-spheroids (1:0) and hetero-spheroids from the 1:0.5 ratio generally exhibited smaller mean diameters ($88 \pm 25 \mu\text{m}$ and $98 \pm 15 \mu\text{m}$, respectively) than those formed from the 1:2 condition ($147 \pm 16 \mu\text{m}$) (**Figure 2.A**). Homo- and hetero-spheroids (1:0.5 ratio) also presented a quite homogeneous size distribution (more than 50 spheroids counted) included in the 50-100 μm range (**Figure 2.C**). The homogeneity level was also significant in the other hetero-spheroid conditions, which effectively stabilized in the 100-150 μm range. This size profile was preserved over time with some changes. On day 10 (**Figure 2.B**), the mean diameter of the various hetero-spheroid conditions slightly increased ($105 \pm 17 \mu\text{m}$, for the 1:0.5 case) and an increased number of bigger hetero-spheroids appeared in certain co-cultures conditions (13 hetero-spheroids out of 100 in the 1:2 condition). Homo-spheroids maintained their mean diameter ($84 \pm 15 \mu\text{m}$) and size homogeneity, and, as a result, no large-size spheroids were identified.

After 10 days of culture, cell viability was still high (>96%) for all conditions (**Figure 5**).

HCM medium

Spheroids presented larger sizes than those obtained in any of the other media used.

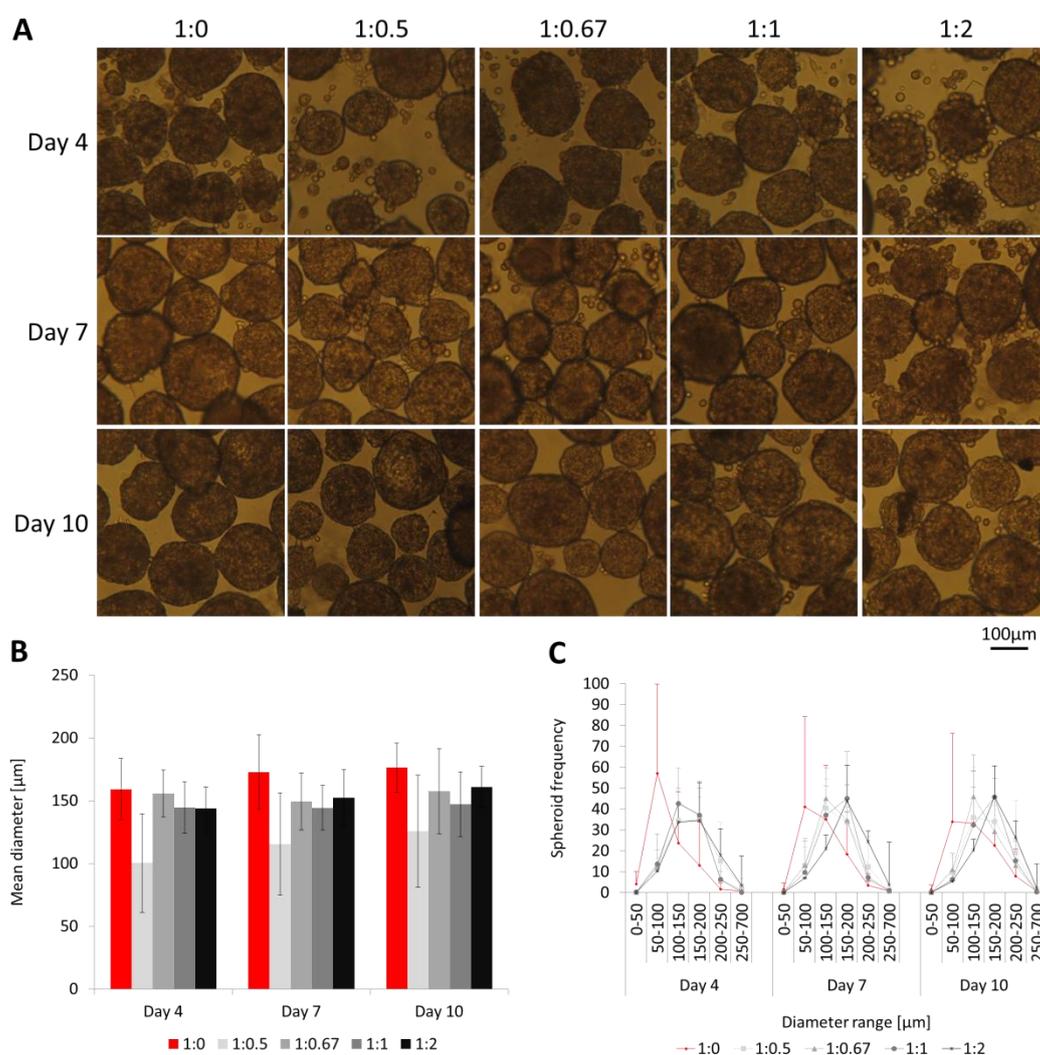


Figure 3. Morphometric evolution of homo- and hetero-spheroids in HCM medium. **(A)** Changes in spheroid size were observed under an inverted light microscope at settled time-points (days 4, 7, and 10). **(B)** Spheroid diameters were measured on the resulting images and used to evaluate the mean value for those obtained from independent experiments ($n=3$). **(C)** Spheroid frequency was evaluated in different size ranges by counting 100 spheroids per condition.

On day 4, homo- and hetero-spheroids presented similar mean diameters (**Figure 3.A**). Hetero-spheroids from the 1:0.5 ratio were the exception, exhibiting tiny sizes ($100 \pm 39 \mu\text{m}$), while the 1:2 condition showed a quantity of hetero-spheroids (18 out of 100) with a large size of 200-250 μm (**Figure 3.C**). On day 10, hetero-spheroids (1:2 ratio) presented an increased number of spheroids (more relevant than in any other condition) with a diameter ranging from 200-250 μm (26 hetero-spheroids out of 100) (**Figure 3.C**). Accordingly, these two culture conditions also underwent a reduction in cell viability on day 10 (**Figure 5**) in comparison with that estimated after four days of culture (data not shown). This decline was more significant in homo-spheroids, and was approximately 30 % on average.

HCM+ECGS medium

Homo- and hetero-spheroids presented similar mean diameters after four days of culture in HCM+ECGS medium. However, slight size differences were observed. The 1:2 condition (with a mean diameter of $135 \pm 44 \mu\text{m}$) already had few (6 out of 100) large hetero-spheroids with a size in the 200-250 μm range (**Figure 4.C**).

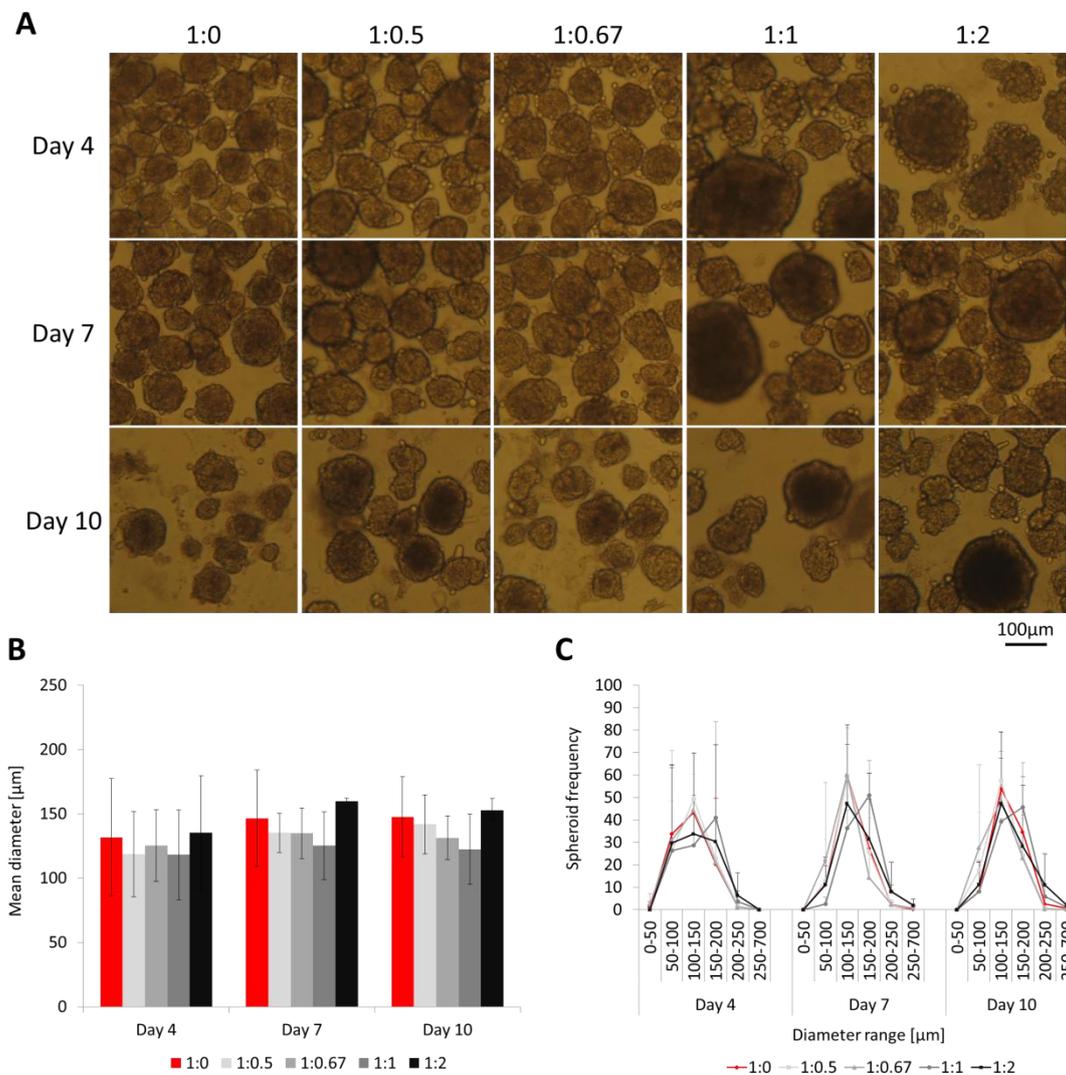


Figure 4. Morphometric evolution of homo- and hetero-spheroids in HCM+ECGS medium. **(A)** Changes in spheroid size were observed under an inverted light microscope at defined time-points (days 4, 7, and 10). **(B)** Spheroid diameters were measured on the resulting images and used to evaluate the mean value for those obtained from independent experiments ($n=3$). **(C)** Spheroid frequency was evaluated in different size ranges by counting 100 spheroids per condition.

A similar observation was obtained for the 1:1 ratio. The other conditions presented a common outcome. Over time, a minor increment in mean diameter was measured in spheroids from all culture conditions. On day 10, the greatest size increase was observed in the 1:2 ratio and those hetero-spheroids had a mean diameter of $153 \pm 9 \mu\text{m}$. In this case, the number of larger spheroids also increased (11 out of 100). An analogous increase was recorded in the 1:1 condition. All the other conditions showed a negligible number of large-size spheroids (<1%) and generally preserved their initial average diameters (**Figure 4**). Cell viability was extremely high after four days of culture (>98%) (data not shown), but was subject to a drop at the end of the culture time (**Figure 5**) which was most noticeable in the homo-spheroids (30-35% in average) and the 1:1 ratio condition (25-20%). However, the other co-culture conditions also underwent a minor reduction in their cell viability.

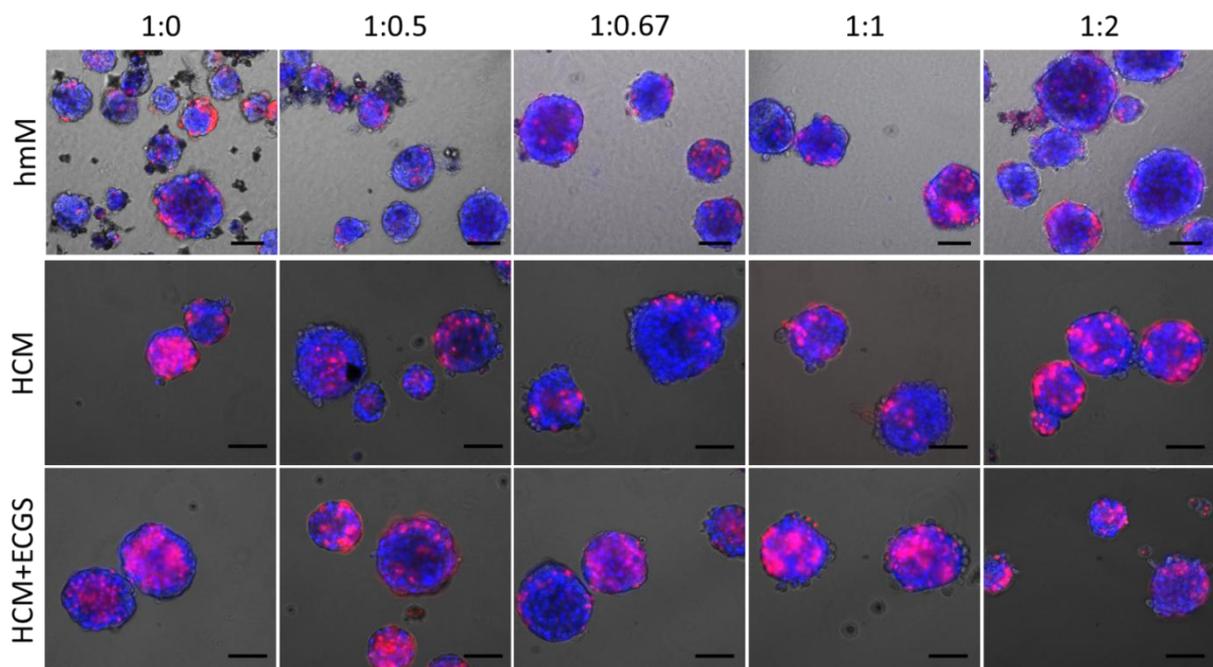


Figure 5. Cell viability of homo- and hetero-spheroids (in hmM, HCM, and HCM+ECGS medium) evaluated on day 10. Cells stained in blue were viable, whereas those in pink identified dead cells. Scale bar equals 100 μm .

3.2. FUNCTIONAL EVALUATION: ALBUMIN PRODUCTION AND UREA SECRETION

Albumin and urea productions are highly differentiated and well-characterized functions of HEPs. They were thus considered here to be indications of synthetic and metabolic hepatic functions capable of identifying the impact of both the presence of NPCs and medium composition on cultured HEPs. Evaluation was carried out at defined time-points (days 4, 7 and 10) and the corresponding results are shown below, separately for each distinct medium.

WE+L-Glu+P/S medium

The presence of NPC-fractions, independently from the co-culture ratio, did not have a beneficial effect on albumin secretion by HEPs (**Figure 6**). The hetero-spheroids showed an unaltered or lesser production of the protein than the homo-spheroids on day 4. After seven days of culture, the situation worsened, and albumin production underwent a dramatic decrease, which was equally significant for both homo- and hetero-spheroids. The cells

already seemed to have lost their functional ability. Albumin assessment was thus not extended over time.

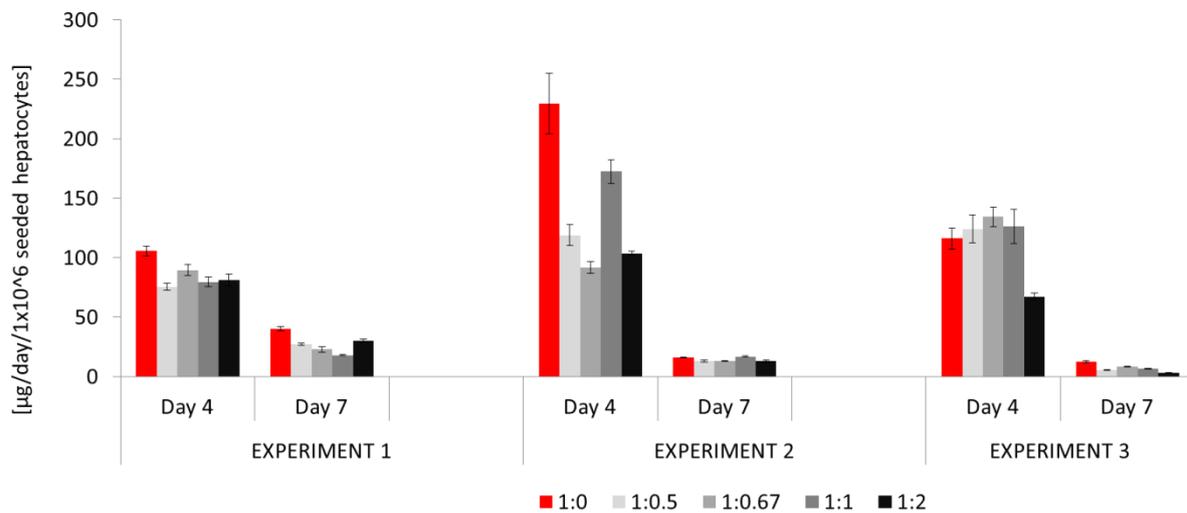


Figure 6. Albumin production of homo- and hetero-spheroids (cultured in WE+L-Glu+P/S medium) on days 4 and 7. The values are presented as a mean \pm SD of triplicate results for the same sample in each independent experiment.

hmM medium

Hepatic functions followed different trends in homo- and hetero-spheroids (**Figure 7**). Dissimilarities were identified between the different co-culture ratios of the hetero-spheroid conditions. An improvement in albumin secretion was already detectable on day 4 in the co-cultures, and was confirmed after seven days (**Figure 7.A**). In correspondence with both these time-points, conditions with a higher density of NPC-fractions (1:1 and 1:2) determined the major enhancement of protein release by the HEPs (on average, 44% and 53% respectively on day 4, and 29% and 52% on day 7, compared to homo-spheroids). On day 10, albumin secretion decreased drastically in all conditions; the hetero-spheroids in the 1:2 condition nevertheless appeared to preserve HEP functionality, with two-fold albumin secretion compared to the homo-spheroids (respectively, 32 ± 15 and 16 ± 3 $\mu\text{g}/\text{day}/1 \times 10^6$ seeded HEPs).

During the initial culture periods (till day 7), the presence of NPC-fractions did not have a great impact on the capacity for urea secretion of the HEPs. Although urea secretion tended to decrease over time (**Figure 7.B**), on day 10, the effect of the co-culture became noticeable and was especially significant in the 1:2 condition, which, on average, was 74% higher than that measured for homo-spheroids.

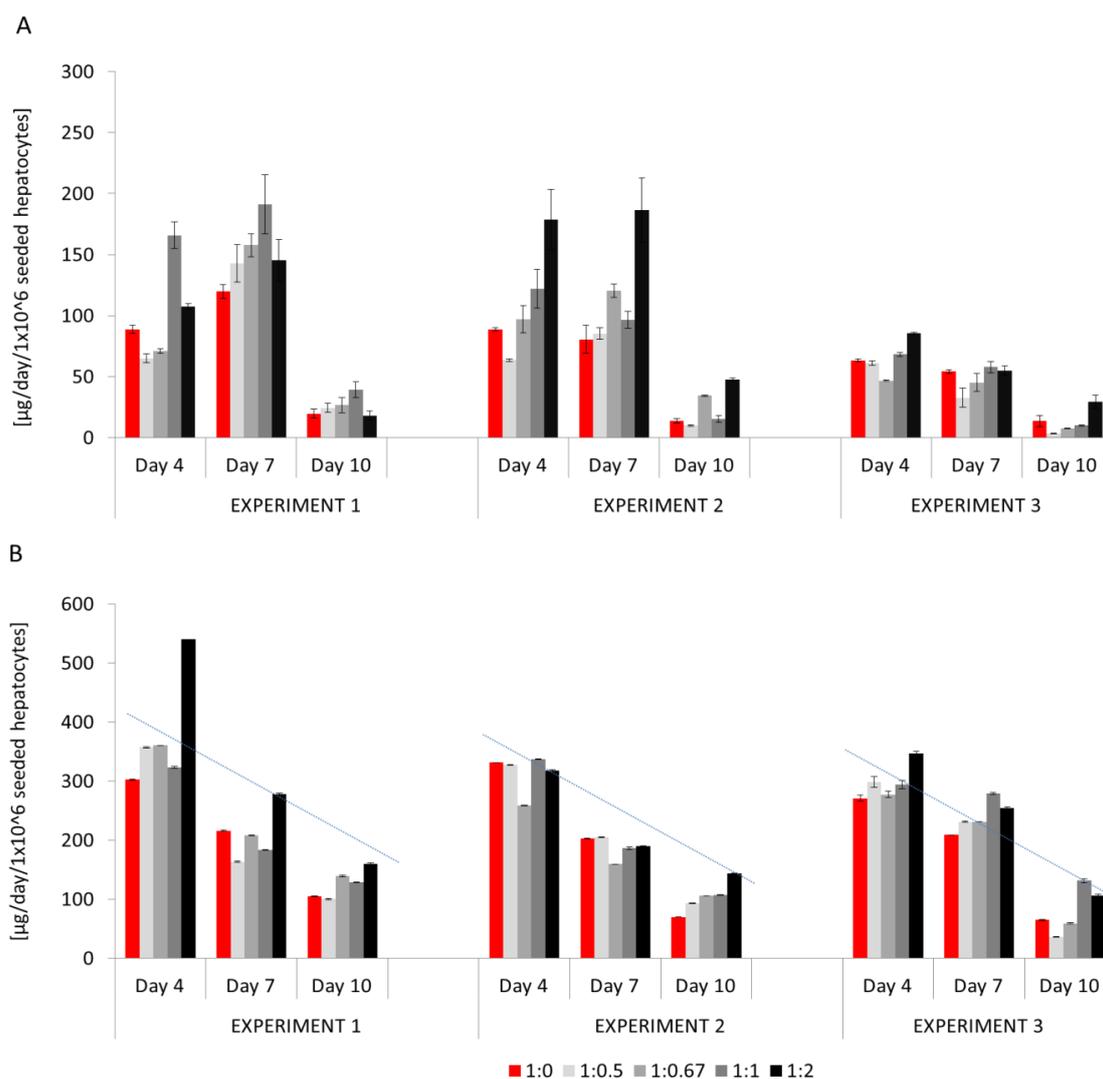


Figure 7. Albumin production (A) and urea secretion (B) of homo- and hetero-spheroids (cultured in hmM medium) estimated on days 4, 7, and 10. Values are presented as a mean \pm SD of triplicate results for the same sample in each independent experiment.

HCM medium

HEP functional secretion responses varied considerably depending on the culture condition. Already on day 4, the presence of NPC-fractions positively impacted the HEPs' ability to produce albumin in hetero-spheroid cases (**Figure 8.A**). At this time-point, conditions with lower NPC densities (1:0.5 and 1:0.67) gave the most relevant increase in albumin production which was, on average, twice as high as that evaluated for the homo-spheroids. Over time, the tendency was inverted. Conditions with a high quantity of NPCs (1:1 and, especially, 1:2) presented significant albumin production which was 3.5- (on day 7) and 3-times (on day 10) higher for 1:2 hetero-spheroids than for homo-spheroids. Urea secretion decreased in all the conditions considered over time and the enhancements were not particularly relevant (**Figure 8.B**).

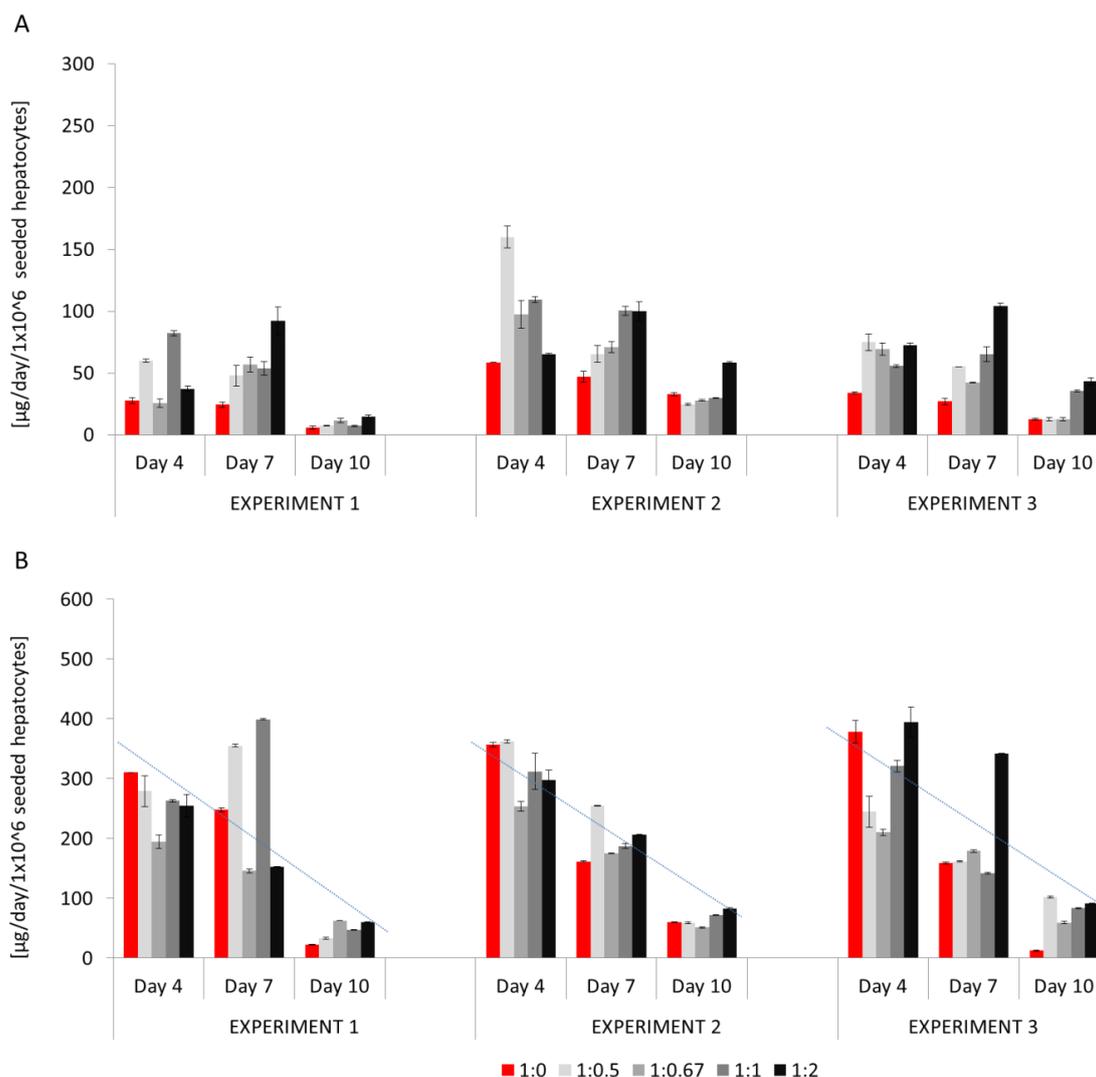


Figure 8. Albumin production (A) and urea secretion (B) of homo- and hetero-spheroids (cultured in HCM medium) estimated on days 4, 7, and 10. Values are presented as mean \pm SD of triplicate results for the same sample in each independent experiment.

HCM+ECGS medium

In HCM+ECGS, HEP potential for producing either albumin or urea was enhanced in both homo- and hetero-spheroid conditions in comparison with that evaluated in the other previous media (Figure 9). However, the presence of NPC-fractions did not seem to affect the secretion of either albumin or urea. A modest improvement associated with co-cultures with higher NPCs densities (1:1 and 1:2) was estimated sporadically in albumin production on day 7. A similar tendency was observed for urea secretion. The increase in the secretion abilities of HEPs in hetero-spheroids could not be appreciated because the HEPs in homo-spheroids already exhibited an increase in their functionalities throughout the culture period (even on day 10). At this time-point, albumin production seemed to stabilize around a mean value (62 ± 4 µg/day/1x10⁶ seeded HEPs) that was common to both homo- and hetero-spheroids (independently of the co-culture ratio). In general, this effect was also evident in urea secretion on day 10.

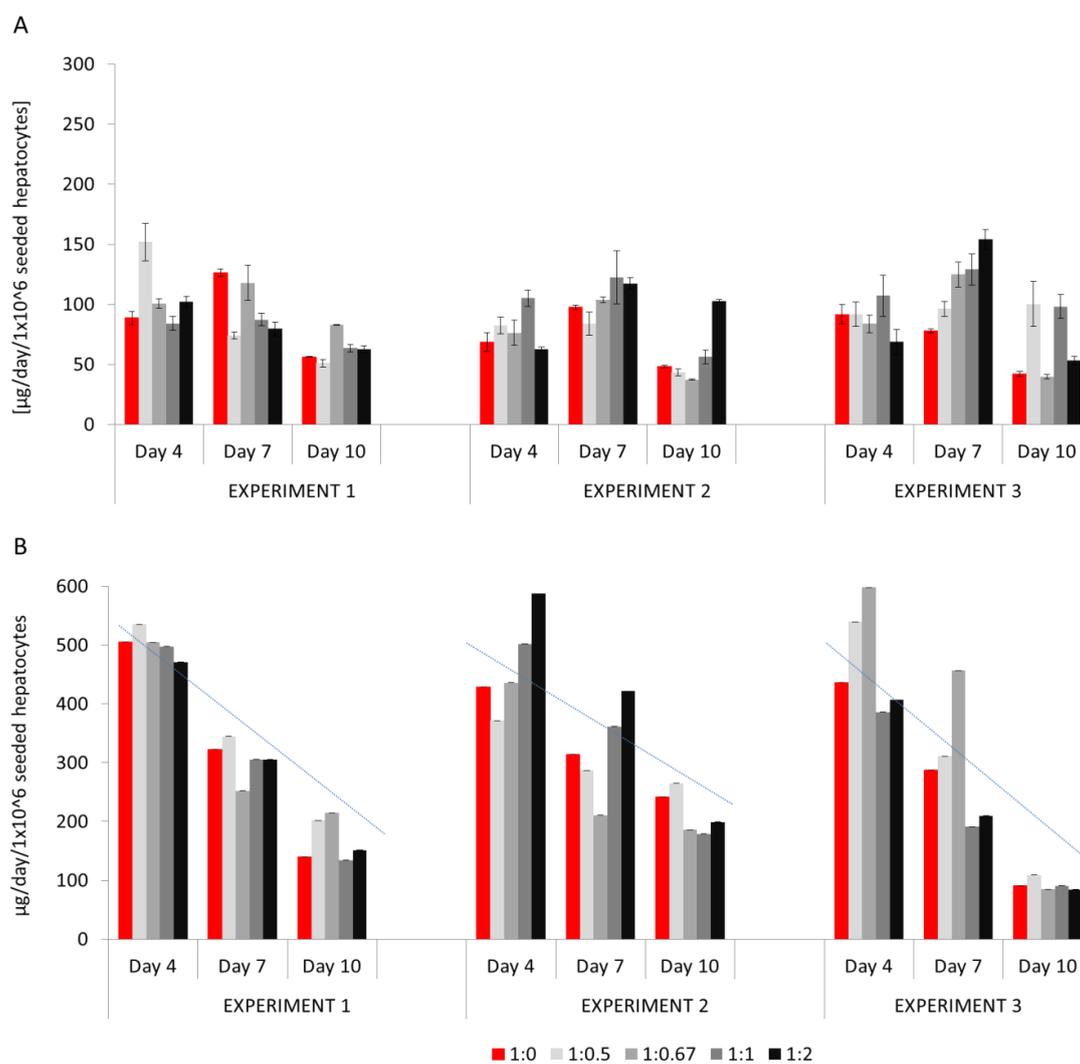


Figure 9. Albumin production (A) and urea secretion (B) of homo- and hetero-spheroids (cultured in HCM+ECGS medium) estimated on days 4, 7, and 10. Values are presented as mean \pm SD of triplicate results for the same sample in each independent experiment.

4. DISCUSSION

Bioengineered models of the liver may be fundamental tools for constructing BAL devices or estimating the toxicological effects of drugs in screening tests. Producing them nevertheless requires that certain obstacles be overcome. These obstacles are associated in particular with the rapid dedifferentiation and function loss of HEPs in *in vitro* cultures. The integration of *in vivo*-like elements (3D organization, the presence of NPCs, and ECM components) into an *in vitro* bioengineered system may provide HEPs with cues reminiscent of the natural environment and that stimulate long-term preservation of their viability and functionality [3, 5, 8, 17, 34]. These factors, however, do not seem to be exclusive when it comes to contributing to HEP preservation. The composition of the culture medium also appears to primarily impact the sustenance of hepatic functions [30]. Combining these culture criteria in the same setting and controlling them with precision may induce a noticeable level of liver-specific functionality in a suitable bioengineered model.

For this purpose, a broad range of culture parameters was taken into account in this study. Primary-enriched hepatic NPC populations (HSCs, LSECs, and KCs) were co-cultured with a series of ratios with HEPs (1:0.5, 1:0.67, 1:1, and 1:2 – HEP:NPCs) in order to mimic the

complexity of the cellular composition of the liver and define an optimal *in vitro* cell proportion. The actual relative cell proportions in the liver (60% HEPs and 40% NPCs) were considered to establish a first ratio of 3 HEPs and 2 NPCs (or 1:0.67, implying 1×10^6 HEPs and 0.67×10^6 NPC-fractions). As *in vitro* cell behavior does not necessarily correspond to *in vivo* behavior, additional ratios were considered by reducing (0.5×10^6) or increasing (1×10^6 and 2×10^6) NPC seeding density with regard to that of the first ratio defined. HEP density was kept constant in all the conditions (1×10^6). Under orbital oscillation, cells were cultured in suspension on non-adherent supports, maximizing the frequency of reciprocal contacts and facilitating massive spheroid formation in a short period. Spheroids conferred a 3D organization on the cells, favoring enhanced establishment of homo- and heterotypic interactions [24, 35] which were, in turn, significantly implicated in improving hepatic functionalities [36]. HEPs seemed to be responsible for spheroid formation, probably thanks to their tendency for self-assembly [37]; in fact, cell aggregation was not observed in cultures of only NPC-fractions in the absence of HEPs (data not shown). Taking into account the difficulty in preserving multiple cell populations in a phenotypical manner in a single environment, four distinct media, differing in their formulations, were assayed for spheroid culture in order to identify which composition made it possible to maintain hepatic functionalities, cell viability, and spheroid integrity over time. From that, we could also indirectly deduce the contribution of both supplemental components and co-culture. Analyses, performed by evaluating albumin and urea production, made it possible to infer the optimal conditions for defining the most adequate bioengineered liver model.

Although all the types of medium induced spheroid formation, maintaining the other parameters depended on the composition of the medium. A poorly-supplemented medium (WE+L-Glu+P/S) did not preserve HEP functions over time, but only allowed HEP albumin production in short-term cultures, in line with previous reports [29]. In these circumstances, the presence of NPC-fractions in the hetero-spheroids was neither effective in secreting higher albumin level in the short-term, nor enough to prevent the rapid loss of the HEPs' ability to secrete albumin in the long-term. Moreover, cell cohesion in the hetero-spheroids was compromised within this standard culture medium and the hetero-spheroids broke down quickly, which was probably exacerbated by the non-self-assembling characteristic of the NPC types. The result of this was that both the NPC populations and the HEPs required a specific medium composition in order to remain active and be able to sustain hepatic functions. Thus, spheroid cultures in a richly-supplemented medium provided interesting insights. The three media contained different constituents particularly implicated in the preservation of HEPs (HCM medium), LSECs (HCM+ECGS medium), and both HEPs and NPC-fractions (hmM medium). Spheroid cultures in HCM and hmM clearly showed the influence of NPC-fractions on improved albumin production, evidence of which was clear after seven days of culture (**Figure 10.A**). The enhancement was NPC-density-dependent and maximized in correspondence with the highest presence of NPC-fractions (1:2 ratio). Logically, the levels of albumin production increased in the hmM medium. The presence of supporting cells was less effective on urea secretion; any noticeable difference was thus identified among the different conditions in the two media (**Figure 10.B**). The effect of the NPC-fractions was almost nil when the spheroids were cultured in the HCM+ECGS medium. Unknown compounds in the ECGS product contributed favorably to increasing albumin and urea production from both homo- and hetero-spheroids, reaching levels greater than those measured in the other cultures (Figure 10). Such secretion values leveled off in homo- and hetero-spheroids and were maintained over the culture time. This outcome was in line with the conclusion of Ijima et al. [30], which stated that medium composition was more important than co-culture for the expression and maintenance of albumin production.

However, the co-culture effect may become relevant in the long-term. This aspect requires further investigation.

With the intention of exploiting these outcomes as a means of defining an actual bioengineered model of the liver, several other facets (cost, variability of the culture conditions) were also taken into consideration and choosing the optimal parameters aimed to minimize them. Accordingly, the HCM medium was chosen because its use could reduce either the variability associated with the lab-self-preparation of the hmM medium, or the additional expenses linked to the purchase of ECGS. HCM medium also sustained the NPCs' functionalities and may have the composition needed for the culture of multiple hepatic cell types. In these conditions, the 1:2 ratio was chosen because it provided optimal preservation of albumin and urea secretion by the HEPs, as well as cell viability in the spheroids. Additional studies need to be carried out to characterize this spheroidal multi-culture system in detail, and to examine a broader spectrum of liver functions than those generally proposed in the literature (albumin production, urea secretion, and cytochrome P-450 biotransformation) in order to better adapt this model to the different requirements.

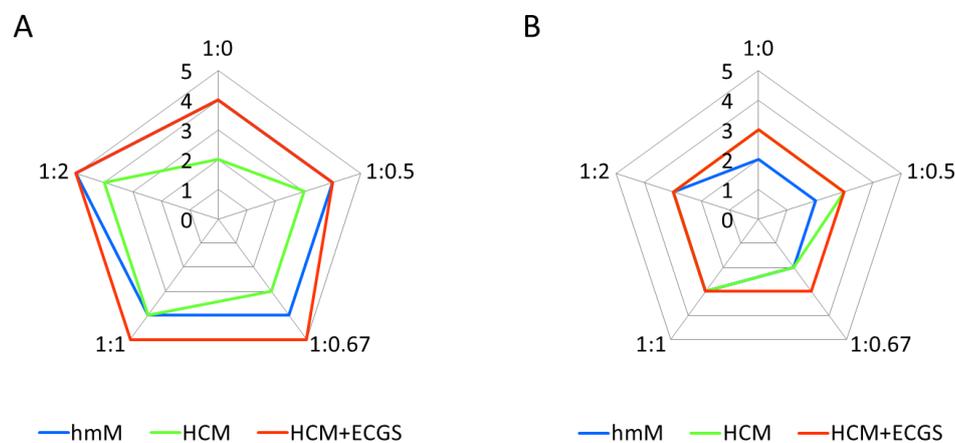


Figure 10. Summary diagrams of (A) albumin production and (B) urea secretion by homo- (1:0) and hetero-spheroids (different HEP: NPC ratios) cultured in the four media used (hmM, HCM, and HCM+ECGS) on day 7. Secretion ranges were identified with a scale of values (0-5) highlighting very low (1), medium (3), and very high (5) productions of albumin and urea.

Homo-spheroids have been considered good candidates for the application of BAL systems [38, 39] because of their ability to preserve liver-specific functions [40-44]. Based on greater improvements and preservation of the hepatic functionalities in our bioengineered model, hetero-spheroids may be more suitable than homo-spheroids for clinical practice. Increment of the hepatic functions may also imply a reduction of the HEP demand for the realization of spheroids to house in the BAL [45].

Spheroid-based drug screening platforms have also been introduced thanks to much closer clinical expression profiles than those seen in two-dimensional models [38, 46]. The inclusion of hetero-spheroids may add value to these models, generating orchestrated reactions to pharmaceutical products which approach the *in vivo*-like manifestations, and permitting long-term toxicity testing by preserving HEP behavior [24, 47].

5. ACKNOWLEDGMENTS

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CHAPTER 4

A SPHEROIDAL MULTI-CULTURE MODEL CONSTITUTED OF HEPATOCYTES, ENDOTHELIAL, KUPFFER, AND STELLATE CELLS FOR PHYSIOLOGICAL *IN VITRO* USAGES

"The microenvironment of the hepatocyte **in vivo** is very important to the maintenance of normal function, including its response to endogenous and exogenous substrates, and can be complex to mimic." – *Valerie Y. Soldatow, 2013.*

ABSTRACT

The need of preserving hepatic functions in *in vitro* applications resulted in the proposition of several solving strategies sharing the final purpose to resemble the *in vivo* environment. Three-dimensional (3D) and co-culture represent key attractive elements in such context. In this study, we present a complex model which combines 3D and co-culture aspects for the culture of primary hepatocytes simultaneously with the three main lining cells of the liver sinusoids (liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells). Specifically, employed cell populations were sorted from the same rat liver and cultured in continuous orbital oscillation for the acquisition of a spheroidal configuration. By means of this model, we aimed to macroscopically investigate the effects of these supporting cells on hepatocyte behaviors in comparison with those manifested by only hepatocytes also cultured in a spheroidal manner. Firstly, the supporting cells impacted on the formation of the spheroids (hetero-spheroids) showing bigger size and rougher surface than spheroids of only hepatocytes (homo-spheroids). Cells occupied preferential locations of the hetero-spheroids reflecting the *in vivo* organization. In this condition, hepatocytes enhanced albumin production and urea secretion over 10 culture days, reaching a peak of albumin release (3-folds higher than the value measured in homo-spheroids) on day 7. On the contrary, hetero-spheroids showed neither an improvement of phase I and II detoxification nor an enhanced polarity of membrane transporters. Though, multidrug resistance-associated protein 3 was only identified in hetero-spheroids. In conclusion, our results indicated the potentiality of this model in structurally reflecting the *in vivo* situation and modulating HEP behaviors. Thereby, it may represent a suitable tool for practical *in vitro* applications.

1. INTRODUCTION

The liver performs over 500 functions which are paramount for the regulation of human body homeostasis [1], including protein, carbohydrate, and lipid metabolism; detoxification of endogenous and exogenous compounds; production of bile for digestion; and secretion of many serum proteins (i.e. albumin, coagulation factors). Because of this functional implication of the hepatic organ, hepatocytes have been largely used in myriad of *in vitro* models addressed to the development of systems for various application areas, such as extracorporeal bioartificial liver (BAL) devices or absorption, distribution, metabolism, excretion, and toxicity (ADMET) screening platforms [2, 3]. However, the hepatocyte usage is limited in time and in terms of functional assessments due to their *in vitro* phenotypical instability; in fact, these cells dedifferentiate and become nonfunctional soon after isolation from their native environment. To preserve the *in vitro* hepatic-specific functionalities for long-term periods, hepatocytes require to be cultured in an *in vivo*-like microenvironment. Several elements have been identified from the *in vivo* context and considered to be paramount to this end (including, extracellular matrix (ECM) components, assisting nonhepatocyte cell types, and a three-dimensional (3D) structure) [4-6]. The spheroidal culture of hepatocytes (forming the so-called spheroids) confers an adequate 3D organization to the cells and, especially, allows restoring more physiological liver conditions [7]. Cellular interactions are maximized in these structures and the hepatocytes survive longer, manifest extended liver-specific functions and cyto-architectural similarities with the *in vivo* configuration [8-13]. In a complementary manner, co-cultures of hepatocytes with supporting non-parenchymal cells not only permit to mimic *in vivo* phenomena but also to emulate the complexity of the liver cellular composition [14]. Hepatocyte functions are

modulated and maintained in long-lasting co-cultures thanks to the establishment of direct cell interactions and/or indirect communication by paracrine substances release (growth factors, cytokines, and ECM elements) [15, 16]. The beneficial contribution of diverse assisting cell types on preserving the *in vitro* hepatocyte functions was testified in numerous two- or three-dimensional co-culture models, generally, through measurements of albumin production, urea secretion, and cytochrome P450 activity [3, 16]. Though, most of the studies defined simplified co-culture platforms, including only one supplemental cell population to the hepatocytes, and, therefore, lacking in the intricate cellular component of the liver. The sole added supporting cell population often possessed a cell line identity (for example, immortalized stellate cells [17]) and/or was not derived from the liver (e.g., rat prostate endothelial cell line [18], human umbilical vein endothelial cells [19, 20], murine 3T3-J2 fibroblasts [21, 22]) being, thereby, devoid of features that are unique to the non-hepatocyte cells of the liver and responsible for certain specific orchestrated responses between hepatic cells (for instance, the peculiar presence of fenestrae on liver sinusoidal endothelial cells involved in filtering size-specific substances before that hepatocytes can access to them [23]). Cellular complexity was improved in a scant number of recent co-culture systems in which primary derived liver non-parenchymal cells (sinusoidal endothelial cell, Kupffer cells, and stellate cells) or non-derived liver cell lines were cultured in combination of two [24-27] or three [28] with hepatocytes. In most of these models, primary supporting cell populations, obtained from different liver donors, underwent a two-dimensional pre-acclimated culture period before their co-culture involvement with the hepatocytes. This approach may lead cells towards dedifferentiation or activation [29, 30] and, therefore, loss of their peculiarities. Alternative models proposed to co-culture hepatocytes with the whole non-parenchymal liver cell fraction after isolation [31-36]. This strategy seems to depict a more realistic way of mimicking the *in vivo* cellular situation; nonetheless, it presents some drawbacks. The supplemental entire non-parenchymal fraction is less controllable in terms of seeding density of its multiple cell constituting components and highly variable due to intra-donors differences in the cell content. Hence, such co-culture approach results to be difficultly transferable among the laboratories.

An *in vitro* system able to recreate the intricate 3D hepatic structure and the multicellular relationships occurring in the liver does not exist at present [4], but it may be useful to overcome the obstacles associated to the *in vitro* hepatocyte phenotypical instability. To fulfill this lack, we define an innovative model that, inspired by the liver intralobular organization, integrates 3D and multiple cellular culture aspects (**Figure 1**). Our system incorporated three major hepatic non-parenchymal sinusoidal populations (sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells) and hepatocytes; the four hepatic cell populations were simultaneously sorted from the same rat and individually seeded in a sole dynamic culture environment according to controlled densities for emulating specific cellular proportions. In these conditions, the different populations aggregated in hetero-spheroids conferring the 3D facet to the global model. Based on the commonly known contribution of the supporting cells in sustaining and modulating hepatic functions [16], we used this complex model to explore the impact of the introduced supporting cells on the hepatocyte behaviors along the time trying, eventually, to infer their ability in recreating more physiological hepatic events in the *in vitro* context. Homo-spheroids (made of only hepatocytes) were considered as control.

2. MATERIALS AND METHODS

2.1. HEPATIC CELL ISOLATION

Hepatic cells were isolated from male Sprague-Dawley rats (Janvier Labs, France) weighting 250-350 g using an elsewhere defined method [37]. Sorting of the non-parenchymal sinusoidal populations was achieved by following previous protocols [38, 39].

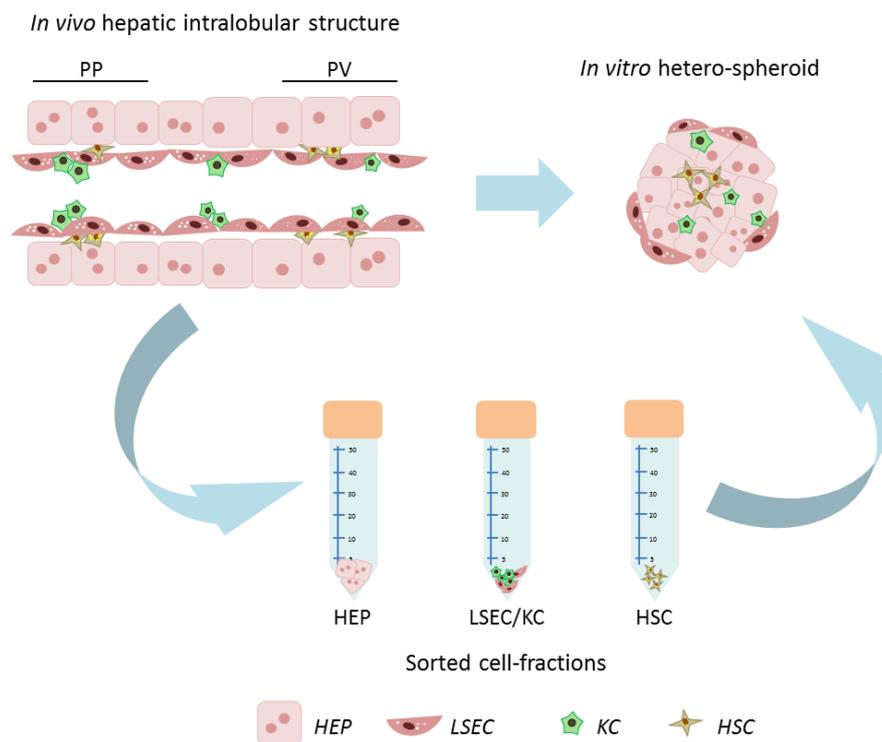


Figure 1. Schematic representation of the concept behind the establishment of the spheroidal multi-cultured model. The proposed model combined 3D organization and cellular composition to emulate the liver intralobular structure and establish a more physiological *in vitro* microenvironment for an improved HEP culture.

In brief, the resulting digested liver cell suspension was centrifuged (50 g, 5 min, thrice) and both pellet and supernatants were distinctly saved from centrifugation steps. The pellet was used to obtain hepatocytes (HEPs). It was suspended in an Easycoll separating solution (Biochrom AG – Merck Millipore) prepared in Dulbecco’s Modified Eagle Medium (DMEM, Gibco – Life Technologies) with final density of 36% and centrifuged (168 g, 20 min). The derived HEP-containing pellet was saved after a wash in DMEM (100 g, 5 min). The three hepatic non-parenchymal sinusoidal populations (NPCs) were sorted from the initial saved supernatants. These latter were split in two portions, each of which was employed to isolate either hepatic stellate cells (HSCs) or a fraction constituted of Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs). To obtain HSCs, supernatants were first repeatedly centrifuged (50 g, 5 min) till no pellet was formed in order to discard the HEP-containing pellets. HEP-free supernatants were centrifuged twice (200g, 10 min) and the resulting HSCs-containing pellets were saved. KCs and LSECs were obtained from the other portion of supernatants. These latter were centrifuged (1350 g, 10 min) and the resulting pellets, collected together, were split in equal volumes and loaded on a density gradient Easycoll separating solution (25-50%), next centrifuged (1350 g, 30 min). Only cells at the interface between the two density cushions were collected, suspended in DMEM and centrifuged (1350 g, 10 min). Final pellet contained KCs and LSECs (termed LSEC/KC-fraction). The entire procedure was carried out at 4°C. Cell viability was assessed by trypan-blue exclusion test.

2.2. ESTABLISHMENT OF THE SPHEROIDAL MULTI-CULTURE MODEL

Isolated hepatic fractions were suspended in hepatic culture medium (HCM™ Hepatocyte BulletKit, Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco – Life Technologies). Suspended cells were inoculated into glass petri dishes (ø x h = 60 x 20 mm) coated with Sigmacote® (Sigma-Aldrich) according to controlled specific densities. The multi-culture condition included HEPs and the different isolated NPC fractions in a ratio of 1 HEP and 2 NPCs (this latter containing 20% of HSC- and 80% of LSEC/KC-fraction). Specifically, 1×10^6 HEPs, 0.4×10^6 HSCs, and 1.6×10^6 LSEC/KC-fraction were seeded in 5 mL of media per petri dish. A mono-culture condition, constituted of only (1×10^6) HEPs, was used as control. Dishes were subject to continuous orbital agitation at 70 rpm with oscillation amplitude of 16 mm (SSL1 orbital shaker, Stuart) in a humidified environment at 37°C and 5% CO₂. Four days after seeding, medium was switched for free-serum HCM. Culture was maintained for 10 days, during which medium was changed every 3 days. Hetero-spheroids formed from the multi-culture condition, whereas homo-spheroids were obtained from the mono-culture one.

2.3. SPHEROID SIZE

Evolution of spheroid size was followed by measuring spheroid diameter (500 counted spheroids per condition) on images acquired under light microscope equipped with a digital camera (on day 4, 7 and 10). Image J Software was used for size measurements.

2.4. ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

Spheroids were collected (on day 4 and 10), rinsed twice in PBS, and fixed in Rembaum solution. Before observation, spheroids were abundantly rinsed in MilliQ water, placed on a sample holder and observed under environmental scanning electron microscope (FEI Quanta 250 FEG) at 15.00 KV of voltage and 4 Torr of pressure.

2.5. CELL VIABILITY

On settled time-points (day 4, 7, and 10), spheroids (about 20 µl) were incubated in a solution of propidium iodide (10 µg/ml) and of Hoechst 33342 (10 µg/ml) in Williams' medium E for 30 min at 37°C under gentle orbital oscillation. Spheroid images were taken under epifluorescence microscope (Leica DMI 6000B, 20x/0.40).

2.6. SPHEROIDS IMMUNOSTAINING AND CONFOCAL MICROSCOPY

On day 10, spheroids were immunostained by following a previous method [40]. Pictures of the samples were taken at different observation plans under confocal microscope (Zeiss LSM 710, 63x/1.40 Oil DIC). Used primary antibodies are listed in **Table 1**, whereas secondary antibodies (Donkey anti-mouse Alexa Fluor® 488 and Donkey anti-goat Alexa Fluor® 594) were purchased from Life Technologies.

2.7. SPHEROIDS HISTOLOGY FOR PERIODIC ACID–SCHIFF (PAS) STAIN

Spheroids were harvested, rinsed in PBS (twice), and fixed in Bouin solution. Spheroids were paraffin-embedded, sectioned at 5 µm, and affixed to charged slides (Superfrost/Plus, Fisher Scientific). Spheroid slides were stained in conformity with the PAS protocol provided with the kit (ref.395B-1KT, Sigma-Aldrich).

Table 1. Primary antibodies used for spheroids immunostaining.

Staining for...	Primary antibody
Cell phenotype	
HEPs	Cytokeratin 18 ⁽¹⁾
HSCs	Desmin ⁽¹⁾
LSECs	SE-1 ⁽³⁾
KCs	CD68 ⁽²⁾
Membrane transport proteins	
	Mrp3 ⁽¹⁾
	Bsep ⁽¹⁾
	Ntcp ⁽¹⁾

⁽¹⁾ Santa Cruz Biotechnology, ⁽²⁾ AbD Serotec, and ⁽³⁾ Novus Biologicals.

2.8. HEPATIC SYNTHETIC FUNCTIONS

Spent media was collected on days 4, 7, and 10 from both conditions, and stored at -20°C. Albumin synthesis was quantified by ELISA test (Rat Albumin ELISA kit, Bethyl Laboratories, Inc.), whereas urea secretion was determined by colorimetric assay (QuantiChrom™ Urea Assay Kit, BioAssay Systems). Analyses were performed in conformity with the manufacturer's instructions.

2.9. URIDINE DIPHOSPHATE GLUCURONOLTRANSFERASE (UGT) ACTIVITY

UGT activity was assayed according to previous defined procedure [41, 42]. In short, spheroids were incubated in 100 µM of 4-Methylumbelliferone (4-MU), prepared William's E medium (devoid of L-glutamine and red phenol, PAN Biotech), for 3 hours at 37°C. Supernatant was saved and used to measure the fluorescence (excitation wavelength of 320 nm and emission wavelength of 450 nm). The 4-MU remaining concentration was deduced from the fluorescence measurements based on a linear standard curve generated in William's E medium.

2.10. ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY

EROD activity was estimated in conformity with previous defined method [43]. Spheroids were incubated in ethoxyresorufin (10 µM), prepared in WE, for 3 hours at 37°C. The substrate included salicylamide (3mM) in order to inhibit phase II enzymes. Supernatant was retained and used to measure the fluorescence (excitation wavelength of 535 nm and emission wavelength of 595 nm) by means of a fluorescence microplate reader (Spectafluor Plus, TECAN). Resorufin formation was deduced from the fluorescence measurements based on a linear standard curve generated with a solution (ethoxyresorufin in WE) containing exogenous resorufin (10 µM).

2.11. INTERLEUKIN-6 (IL-6) LEVELS

Spent medium, retained after spheroids incubation in 4-MU solution, was used to measure the level of released IL-6 (on day 4, 7, and 10) in both homo- and hetero-spheroid conditions. This secretion was compared to that of homo- and hetero-spheroids cultured in standard William's E medium. Estimation was performed by using the rat IL-6 ELISA kit (Sigma-Aldrich) in accordance with the manufacturer's instructions.

2.12. STATISTICAL ANALYSIS

Results were presented as mean \pm SD of three independent experiments (n=3). Statistical significance was determined using nonparametric Mann-Whitney test by means of Prism - GraphPad tool (considering two-tailed test and confidence interval of 95%). P-values <0.01 were considered to be significant.

3. RESULTS

3.1. MORPHOMETRIC CHARACTERISTICS AND VIABILITY OF HOMO- AND HETERO-SPHEROIDS

To define the spheroidal multi-culture model, we established a robust protocol to isolate the four main hepatic (parenchymal and non-parenchymal) populations simultaneously from the same rat liver. Sorted hepatic populations, cultured in a controllable multi-culture condition (1 HEP and 2 NPCs, this latter constituted of 20% of HSC- and 80% of LSEC/KC-fraction), aggregated under orbital oscillation and formed hetero-spheroids. Similarly, HEPs generated homo-spheroids when cultured in absence of NPC-fractions. Hetero- and homo-spheroids were produced in massive quantities and in short time (3 culture days) thanks to our oscillation culture strategy that permitted to increase the frequency of contacts between suspended cells facilitating their spontaneous assembling. Hetero- and homo-spheroids showed size differences during the culture period. Hetero-spheroids presented higher mean diameter (152 ± 13 , 169 ± 29 , and 173 ± 24 μm respectively on day 4, 7, and 10) than homo-spheroids with a difference of 52, 48, and 38% respectively at day 4, 7, and 10 (**Figure 2.A**). Spite of a slight size increase in both spheroid conditions along the time, mean dimensional growth was generally more important for homo- than hetero-spheroids (respective augmentation of 26% and 14% between days 4 and 10) (**Figure 2.A**). Much frequently, hetero-spheroids presented larger diameters (60, 120 and 130 spheroids larger than 200 μm out of 500, respectively on day 4, 7 and 10) compared to homo-spheroids.

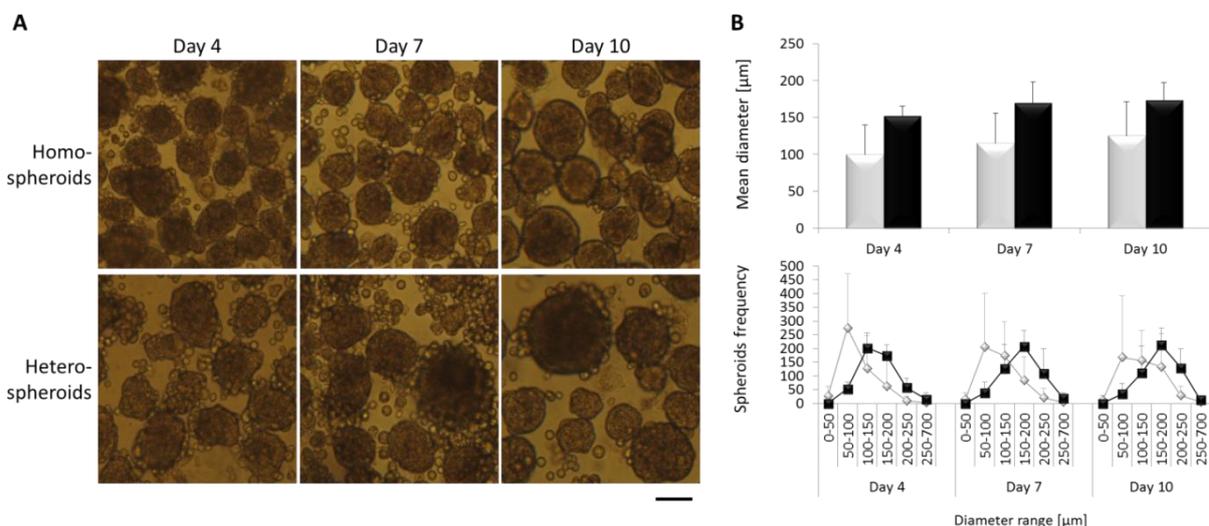


Figure 2. Characterization of homo- and hetero-spheroids. (A) Morphology and (B) size were followed along culture time. Grey and black bars indicate homo- and hetero-spheroids, respectively. Scale bar equals to 100 μm .

However, frequency of large homo-spheroids (> 200 μm in mean diameter) also incremented with time (from 11 to 33, respectively on days 4 and 10).

Morphology was also distinctive for the two spheroid categories and underwent modifications along the time. On day 4, hetero-spheroids exhibited a rougher surface and, likely, a less compactness in comparison with the homo-spheroids (**Figure 3.B**). These

differences were still evident on day 10, although the hetero-spheroids were more compact showing smoother surfaces (**Figure 3.B**). Pores appeared on surfaces of both hetero- and homo-spheroids.

Viability revealed size-independent tendency (**Figure 3.A**). Cells, which were well alive on day 4 (95-98%) in both conditions, experienced a drop of their viability on day 10. This was especially remarkable in homo-spheroids which, despite their smaller size ($125\pm 46\mu\text{m}$ compared to $173\pm 24\mu\text{m}$ for hetero-spheroids), showed a reduced mean viability to about 65-70% with dead cells randomly localized in their body rather than placed in the core. On the contrary, hetero-spheroid viability remained high ($\geq 85\%$) on day 10.

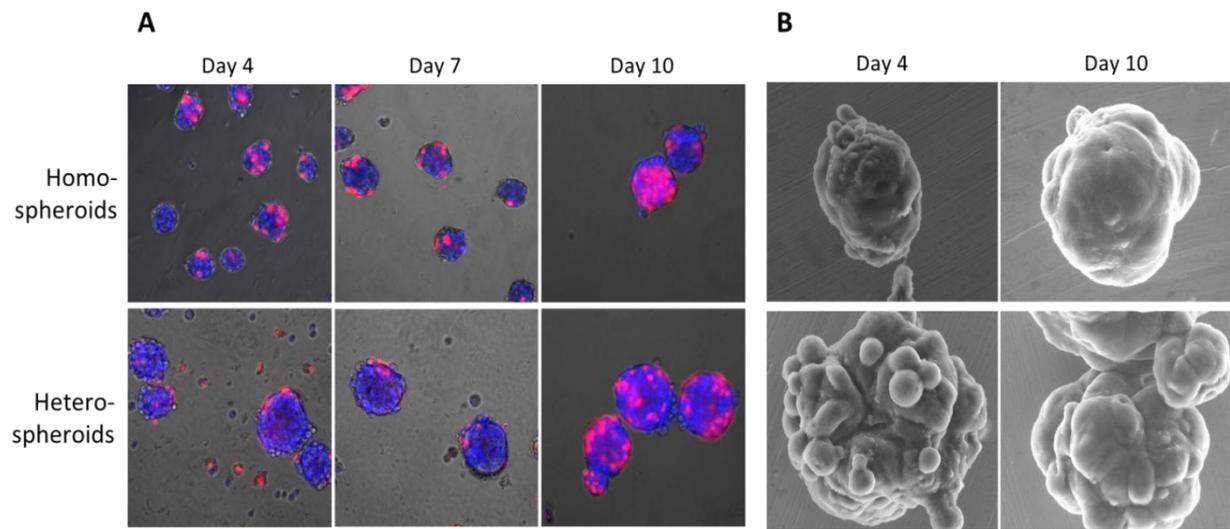


Figure 3. Characterization of homo- and hetero-spheroids. **(A)** Viability and **(B)** compactness of homo- and hetero-spheroids were observed on days 4, 7, and 10. Scale bar equals to 100 μm .

3.2. PHENOTYPES' PRESENCE AND LOCALIZATION IN THE SPHEROIDS

To determine whether the various seeded NPC populations (HSCs, KCs, and LSCs) were still present in the hetero-spheroids after ten days in culture and confirm that any NPC contamination was detected in homo-spheroids, spheroid-constituting cells were immunostained with antibodies specific for each population type. **Figure 4.A**, which shows the spheroids mid-sagittal plan, evidenced the preservation of cells' phenotypes and a clear difference between hetero and homo-spheroids in terms of cell composition. Hetero-spheroids exhibited a noticeable NPC content. Every NPC population seemed to occupy preferential locations of the hetero-spheroids (**Figure 4.B**). HSCs were especially observed in the core of the hetero-spheroids forming crossed circular configurations by establishing connections between them. KCs appreciated several areas of the hetero-spheroids being placed inside as well as superficially. LSCs predominantly covered the surface or, however, were situated closely the external side of the hetero-spheroids. Lastly, HEPs resulted to be the framework of the spheroidal architecture in both hetero- and homo-spheroids. Homo-spheroids possessed negligible presence of NPCs.

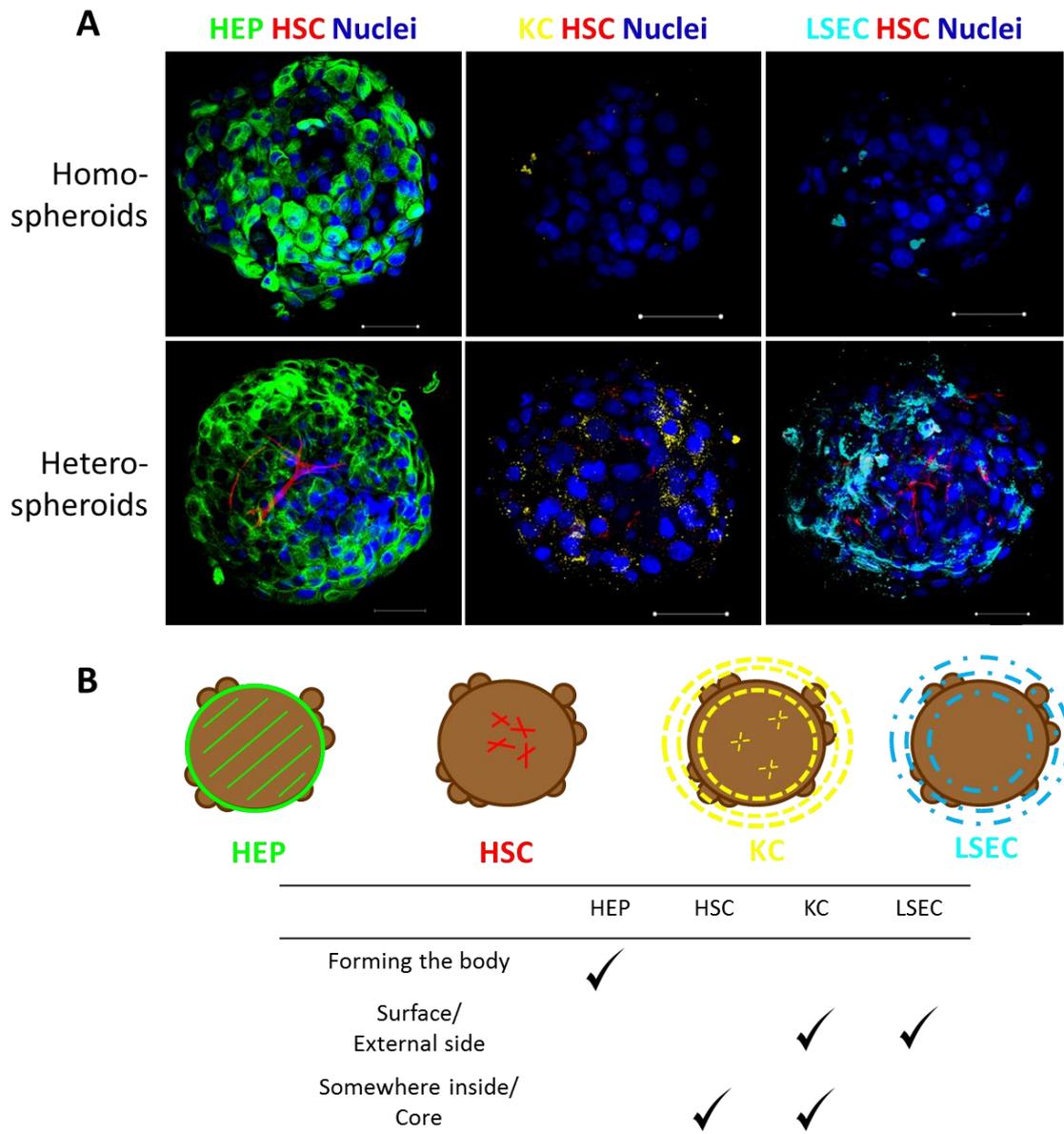


Figure 4. Characterization of the cell phenotypes in homo- and hetero-spheroids. **(A)** Spheroid-constituting cells were immunostained and spheroid mid-sagittal plan was observed under confocal microscope (scale bar=50µm). **(B)** Schematic representation of cell localization in the hetero-spheroids.

3.3. CELL POLARIZATION

HEPs' ability to preserve their characteristic polarity was investigated by staining homo- and hetero-spheroids with specific antibodies for some fundamental transport proteins implicated in phase 0 and III of the hepatic biotransformation/detoxification processes. As shown in **Figure 5**, positive-staining for both Bsep and Ntcp was detected in both homo- and hetero-spheroids. The two transporter proteins were similarly distributed in both conditions, but differently localized through the spheroid body. Bsep staining was especially situated in the spheroid inner area; whereas NTPC was identified in the more external spheroid layers showing a weaker expression compared with that of Bsep. However, the staining was sporadic, less insensitively expressed, and morphologically imprecise in hetero-spheroids than in homo-spheroids. On the other hand, only hetero-spheroids exhibited Mrp3 positive staining (**Figure 6**).

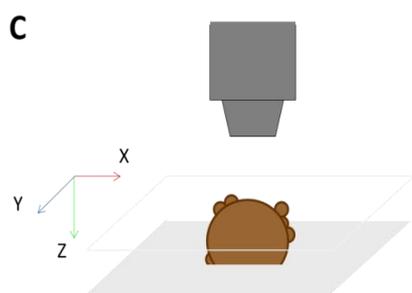
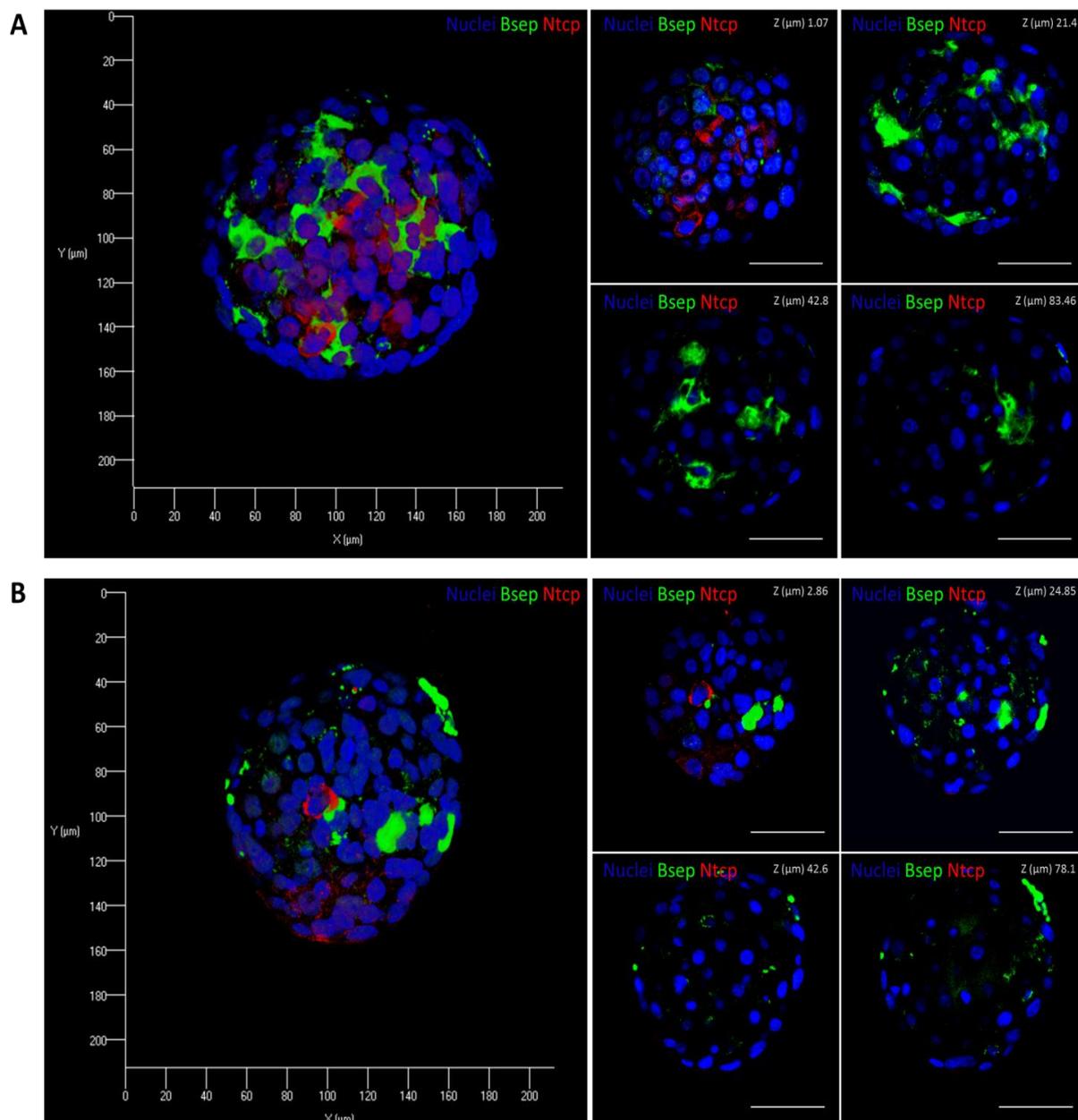


Figure 5. Bsep and Ntcp existence in homo- and hetero-spheroids. Basolateral (Ntcp) and apical (Bsep) bile transporter proteins were stained in both **(A)** homo- and **(B)** hetero-spheroids and observed at different sagittal plans of their structure. These images were snapped under confocal microscope and 3D image reconstruction was obtained from spheroid stacks by using ZEN 2009 light edition software. (Scale bar=50 μ m). **(C)** Sketch of the sample orientation during observation.

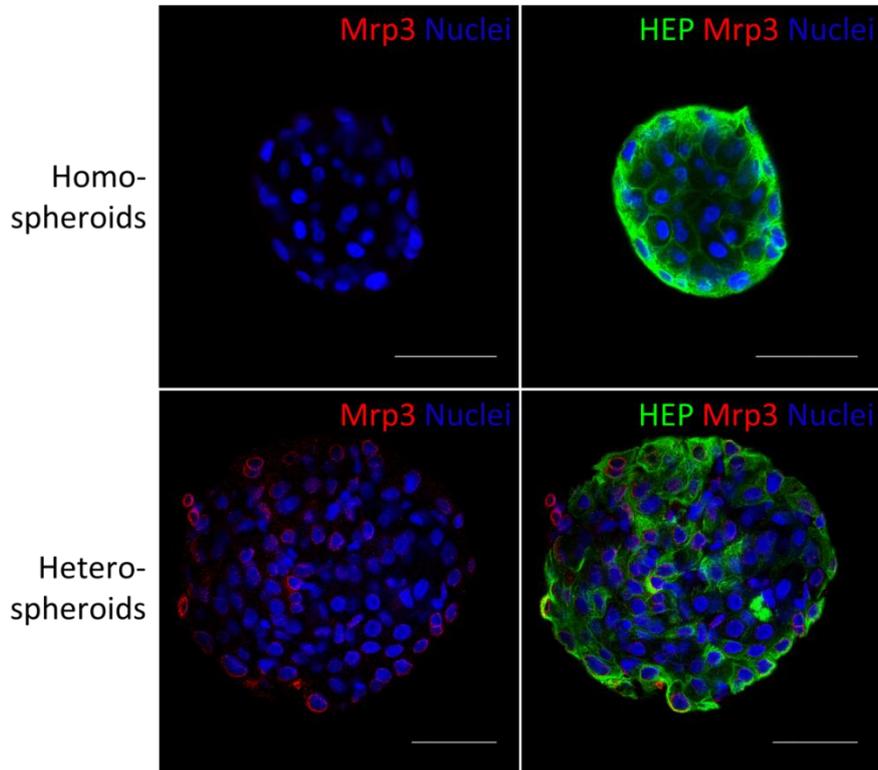


Figure 6. Mrp3 presence in homo- and hetero-spheroids. Spheroids were immunostained with Mrp3 antibody to identify the existence of the corresponding basolateral transporter on hepatocytes. To better localize this staining, hepatocytes were also stained with their specific cytokeratin 18 antibody. Scale bar equals to 50 μ m.

3.4. GLYCOGEN STORAGE

Glycogen was lightly stored in both hetero- and homo-spheroids; however, it was likely more abundant in few sporadic hetero-spheroids (**Figure 7**). On the contrary, homo-spheroids exhibited bright punctuate staining (black arrows in **Figure 7**) that was reminiscent of different other proteins' staining in the normal liver (e.g. anti-trypsin).

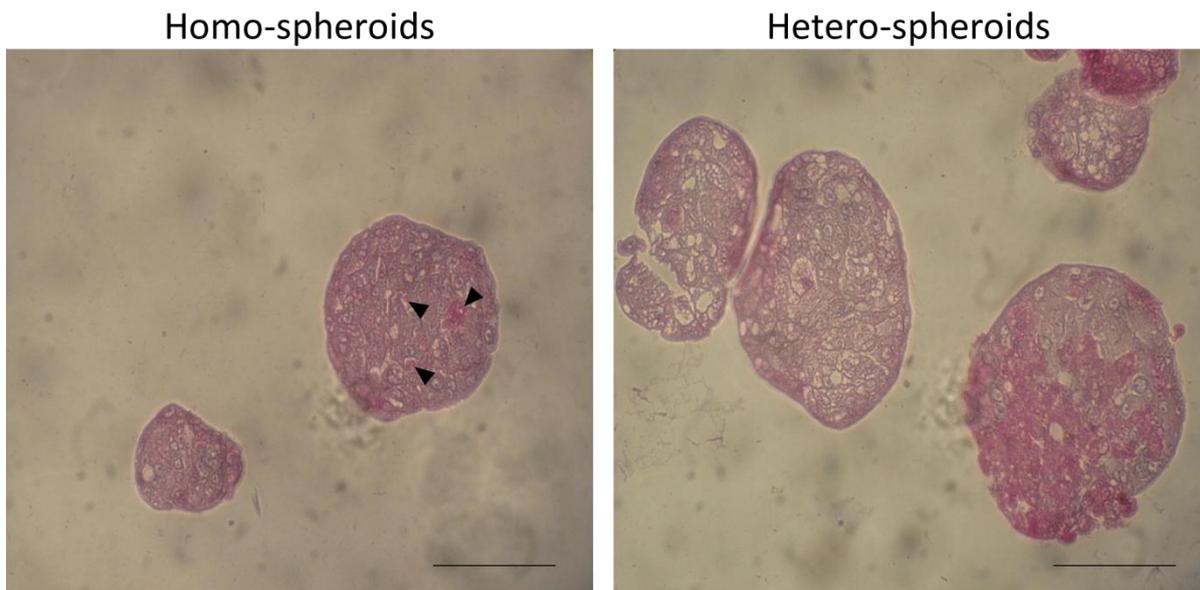


Figure 7. Glycogen storage in homo- and hetero-spheroids on day 10. Scale bar equals 100 μ m.

Other differences were detected between homo- and hetero-spheroids on the histology slides. Namely, these latter showed an internal structure characterized by the presence of a series of opened, interconnected holes which may be due to the placement of lipids within the hetero-spheroids. These may have exploded during sample freezing and, hence, left cavities. Lipids may, in addition, result from the stock in quiescent HSCs. These aspects require further investigation.

3.5. ASSESSMENT OF HEPATOCYTE SPECIFIC-FUNCTIONS

To establish whether the presence of LSECs, KCs, and HSCs in hetero-spheroids beneficially impacted on hepatocyte functionality, some hepatic activities were investigated over the entire culture time and compared with homo-spheroids measurements.

Albumin and urea productions were considered as indexes of metabolic and synthetic activities of the HEPs (**Figure 8**). Both functions were more important in hetero- than in homo-spheroids. Already on day 4, hetero-spheroids manifested augmented levels of albumin (1.45-fold higher) than those of homo-spheroids. Such difference in albumin production became even more remarkable with time (on day 7 and 10) reaching values that were approximately 3-fold superior than those of homo-spheroids. Furthermore, on day 7, albumin production underwent an increment in the only hetero-spheroid condition (from 58 ± 18 , on day 4, to 99 ± 6 $\mu\text{g/day}/1 \times 10^6$ seeded hepatocytes, on day 7). By contrast, homo-spheroids maintained about constant levels of albumin secretion over a culture week (40 ± 16 and 33 ± 12 $\mu\text{g/day}/1 \times 10^6$ seeded hepatocytes respectively on day 4 and 7) which, then, underwent a drop of about 50% on day 10. Although urea secretion tended to decrease with time, hetero-spheroids also exhibited elevated urea secretion on day 7 and 10 in comparison to that of homo-spheroids (respectively 1.23- and 2.48-times more boosted). However, the effect of the NPC populations in hetero-spheroids was less important on the urea secretion ability of the HEPs.

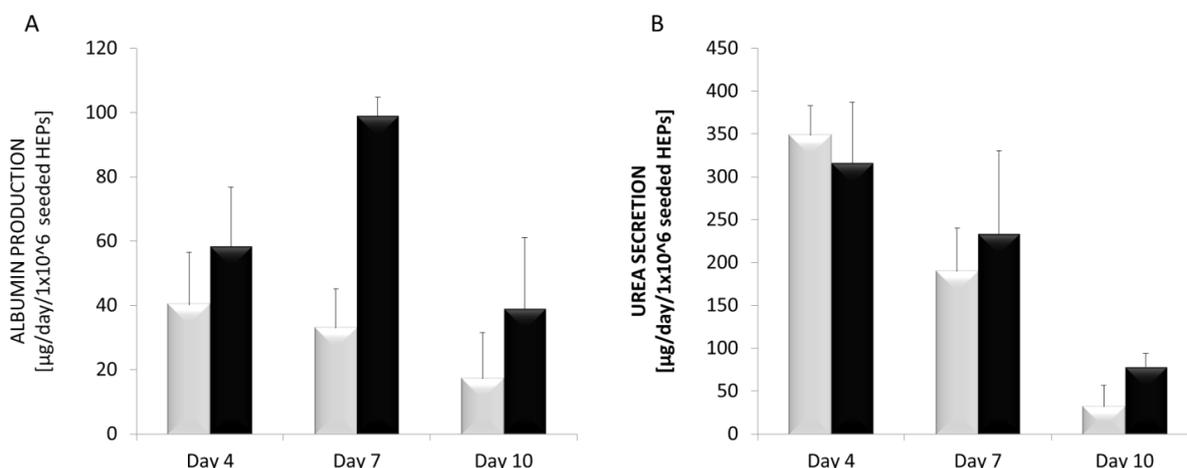


Figure 8. Synthetic hepatic functionalities, albumin (A) and urea (B) productions, of homo- and hetero-spheroids. Grey bars represent homo-spheroids, black ones symbolize hetero-spheroids.

Phase I and phase II biotransformation functions were also evaluated. Here, the presence of the supporting cells did not beneficially affect the *in vitro* detoxifying activities of the HEPs. In fact, hetero-spheroids were less apt to convert ethoxyresorufin into resorufin (phase I, EROD activity) over the entire culture time as compared with homo-spheroids (**Figure 9.A**). Similarly, hetero-spheroids manifested a slight reduced (on day 4) or approximately equal ability (on day 7 and 10) in metabolizing the 4-MU substrate than homo-spheroids (**Figure 9.B**).

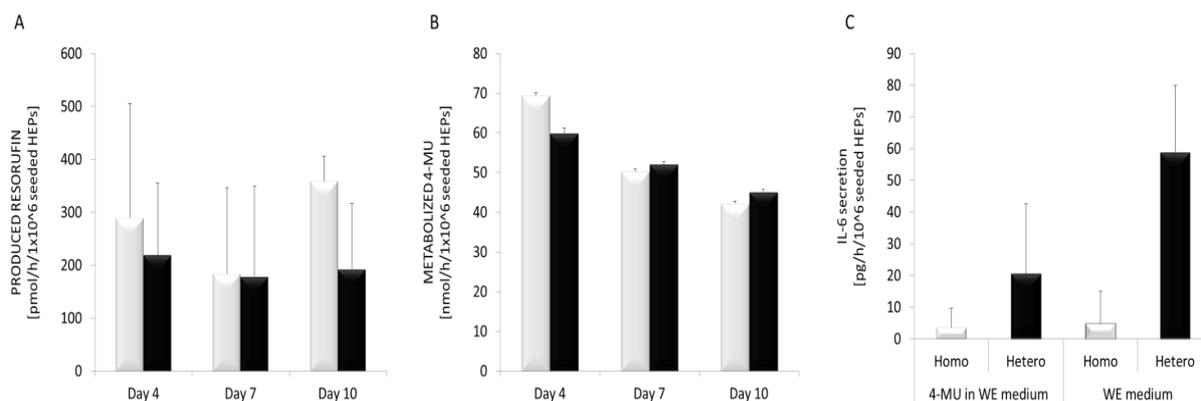


Figure 8. Assessment of (A) phase I (EROD) and (B) phase II (UGT) biotransformation activities of homo- (grey bars) and hetero-spheroids (black bars). Interleukin-6 (IL-6) secretion (C) was also evaluated in (grey bars) and hetero-spheroid (black bars) conditions incubated in presence or not of 4-MU substrate. Graph is referred to measurements on day 4.

3.6. IL-6 SECRETION

To discriminate the influence of the NPC populations on the biotransformation function of the HEPs in the hetero-spheroids, we examined the presence of IL-6 in supernatants collected from both spheroid groups cultured in presence or not of 4-MU substrate. A basal secretion of IL-6 was only detected in hetero-spheroid conditions after three hours incubation. IL-6 was revealed uniquely on day 4 independently from the two used culture solutions (Figure 9.C).

4. DISCUSSION

The hepatic lobule is the structural unit of the liver. The intralobular architecture presents overlapping of one-cell-thick plates constituting of HEPs which are, in turn, spaced by hepatic sinusoids. The hepatic sinusoids are peculiar blood capillaries of the liver formed by LSECs and especially inhabited by KCs and HSCs. Taking into account this *in vivo* internal organization of the hepatic lobule, we recreated an *in vitro* spheroidal multi-culture model which considered both the *in vivo* three-dimensional arrangement and variety of the hepatic cellular component. By the inclusion of these elements, the model intended to reproduce an *in vitro* liver tissue-like microenvironment that more suitably could house HEPs and maintain their functionalities in long-lasting cultures for disparate application fields.

Our *in vitro* spheroidal multi-culture model was composed of primary LSECs, KCs, and HSCs in addition to primary HEPs. The four hepatic cell populations, sorted from the same rat liver and then cultured together under orbital agitation, self-assembled in form of spheroids. Beyond a closer reflection of the *in vivo* 3D physiological situation [7], spheroidal organization generally permits to maximize the establishment of complex cell-cell interactions which seem to be necessary to induce stable liver-specific functions in culture [16]. Thus, in order to verify the dependability of our *in vitro* spheroidal multi-culture model in terms of functional stabilization of the HEPs, we investigated several hepatic specific activities and inferred the influence of the above mentioned supporting cell populations on diverse HEP behaviors and features over time.

We previously deduced the pivotal role of the HEPs in building up the spheroids (see Chapter 3) likely associated to their spontaneous self-aggregation tendency [44]. Still, the presence

of NPC-fractions affected the morphometric characteristics of the formed spheroids. In fact, hetero-spheroids were dissimilar to homo-spheroids, exhibiting rougher surface and bigger size during the entire culture period. The distinct rough aspect of the hetero-spheroids may be principally ascribed to the LSECs that may continue to manifest their shape even though they were well-integrated with the rest of the aggregate body. LSECs, in fact, especially localized on the external layer, as also elsewhere reported [18], probably forming a barrier for the interior spheroid-compartment as takes place in the liver [45, 46]. Hetero-spheroid core showed HSCs that, alike the native organ where they spread their long cytoplasmic processes into two or more neighboring sinusoids [47, 48], dispersed their extensions through the hetero-spheroid thickness forming intricate, often circular in shape, arrangements. Similarly, the multi-positioning of the KCs in various districts of the hetero-spheroids may result from their *in vivo* motile ability [49, 50] that may impede the cells to settle down in specific regions of the aggregates. Hence, the integration of supporting NPC types conferred a superior *in vivo*-like complexity to the model in terms of cellular composition and localization.

Among the enormous set of hepatic functions, we focused on synthetic and phase I/phase II biotransformation metabolic activities of the HEPs as these may be conformed to diverse requirements of disparate *in vitro* applications. By means of such different functions, we could, in addition, better scrutiny the involvement of the supporting NPC types on regulation of HEP duties. Assisting NPC populations impacted on HEP functionalities. On the one hand, these cell types were responsible for the enhancement of synthetic functions (albumin and urea secretion) of the HEPs in the hetero-spheroids. Such improvement may be associated with the establishment of heterotypic interactions in the spheroidal structure [16, 22, 51]. However, this aspect requires additional analysis. On the other hand, supporting cells did not have any beneficial effect on phase I (EROD) and phase II (UGT) activities. The detoxifying activity, evaluated by EROD test, of the HEPs in co-culture conditions has been previously reported [33, 52-54]; many groups stated the beneficial impact of assisting cell types on improving such function. For instance, Chia et al. stated that mouse embryonic fibroblasts enhanced cytochrome P-450 1A1/2 function of preformed HEP spheroid likely by means of release of soluble factors and establishment of heterotypic cell-cell interactions [52]. Instead, UGT functionality has been sparsely evaluated in previous hepatic co-culture models. Nonetheless, Leite et al. demonstrated a long-term maintenance and improvement of UGT activity in a spheroidal co-culture model of mouse embryonic fibroblasts and rat HEPs [42]. Our inverse outcomes may be due to the cellular composition of the NPC component that may have reacted at the presence of the substrate by releasing molecules which, in turn, may have negatively affected the HEP biotransformation potentiality in hetero-spheroids. The role of KCs in secreting pro-inflammatory cytokines (such as, tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6) and their consequent involvement in the downregulation of xenobiotic metabolizing enzymes (i.e. cytochrome P-450 (CYP) and UDP glucuronosyl transferase (UDPGT)) has been amply documented [55, 56] also *in vitro* culture studies of KCs and HEPs [57-59]. Although the mentioned effects have been re-produced in *in vitro* inflammatory-induced settings (e.g. under stimulation with lipopolysaccharide (LPS)) which emulated the *in vivo* situation, Hoebe et al. demonstrated that KCs strongly suppressed the activity of at least CYP3A and UDPGT even in the absence of LPS stimulation [57]. In accordance with this finding, we evaluated the release of IL-6 in homo- and hetero-spheroid cultures in presence or not of 4-Methylumbelliferone drug. Our data revealed IL-6 production by the only hetero-spheroids at the very beginning of the culture period. Accordingly, the initial reduced biotransformation (phase II and, likely, phase I) activity of the HEPs in hetero-spheroids may be due to the secretion of IL-6 by a specific NPC type or via

interplay between several of them. However, the release of IL-6 may not be correlated with the presence of the 4-MU substrate since it was also revealed in absence of the molecule, but rather to an early activation of the KCs which may have required a longer acclimation culture time from isolation procedure [3]. However, further analysis must be carried out to scrutiny a broader range of soluble factors secreted by the diverse NPC populations and get insight about their implication in the phase I and phase II biotransformation functions of the HEPs in our spheroidal multi-culture model.

Membrane transport proteins play a major role in biotransformation and detoxification of drugs as they allow uptake of xenobiotics (and other compounds, including bile acids) from blood in HEPs (sinusoidal or basolateral transporters) and excretion of drugs or their metabolites from HEPs into bile canaliculi (canalicular or apical transporters) or bloodstream [60, 61]. Among disparate transporters, HEPs exhibit the efflux pump called multidrug resistance-associated protein 3 (MRP3 in human and Mrp3 in rat) that is especially involved in the outflow of drug metabolites from the HEPs into the bloodstream permitting their systemic exposure [62]. This transporter is localized at the basolateral pole of the human HEPs, whereas, its position in rat HEPs is subject of controversies [63]. Though, it has mainly individuated at the sinusoidal side in rat HEPs. Hepatic transport of bile acids is primarily mediated by the Na⁺-tauro-cholate cotransporting polypeptide (Ntcp), located at the sinusoidal side, and the bile salt export pump (Bsep), placed at the apical pole of the HEPs [3, 61]. Hepatocyte polarity is unlikely maintained in standard *in vitro* culture, which, indeed, is better preserved in 3D culture condition [64]. In conformity with that, Bsep and Ntcp transporters were identified in both homo- and hetero-spheroids in spite of a different expression in the two conditions. Such dissimilarity highlighted a reduced expression of both proteins in the hetero-spheroids which, in line with the above statements, may be consequence of mediators secretion by NPCs implicated in the downregulation of HEP membrane transporters [65, 66]. By contrast, Mrp3 transporter was exclusively identified in hetero-spheroids.

To conclude, our results indicated the potentiality of this model in structurally reflecting the *in vivo* situation and modulating HEP behaviors. Thereby, it may represent a suitable tool for practical *in vitro* applications, such as BAL devices. The NPC populations' environmental sensing may perfect the susceptibility of the model so that HEPs could more adequately react to disparate external insults and trigger more physiological events. A more suitable HEP performance may entail a reduced demand of these hepatic cells and an easier development/usage of the BAL in clinical field. The current outlook of this approach is promising even though much remains to be elucidated. In this regard, it may be relevant to evaluate additional hepatic-specific functions (including ammonia detoxification, which is an impaired function in both acute and chronic liver failure patients and primarily responsible for hepatic encephalopathy [67-69]) for longer culture periods and to explore the influence of NPC populations in cooperating with the HEPs in the performance of these activities. Furthermore, it may be worth investigating more specific NPC functionalities in order to, hopefully, appreciate the adaptability of the model to several other *in vitro* mimicking situations. For instance, drug screening tests may require the presence of KCs in a liver model in order to evaluate the toxicity of certain products in a more physiological manner. Accordingly, KCs may more suitably react to inflammatory stimuli, which correlated with the drug delivery may induce toxicity, and complementary trigger a cascade of other HEP functions (e.g. acute phase response [70, 71]).

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CHAPTER 5
HEPATIC MULTI-CULTURED SPHEROIDS
MICROENCAPSULATED IN ALGINATE BEADS

"...the microencapsulation of intact cells... the enclosed material might be protected from destruction and from participation in immunological processes, while the enclosing membrane would be permeable to small molecules of specific cellular product which could then enter the general extracellular compartment of the recipient." – Thomas M. S. Chang, 1962.

ABSTRACT

Liver cell transplantation and bioartificial liver devices require suitable cellular platforms enabling hepatocytes to survive and perform specific functions for temporary treatment of liver failure patients. Alginate-based-encapsulation of hepatocyte spheroids has been considered as promising strategy to enhance the preservation of liver functionalities *in vitro*; however, it lacks crucial elements related to the complexity of liver cellular component. In this study, we presented a novel culture approach for liver tissue engineering applications which integrated three major hepatic non-parenchymal populations in the microenvironment of the alginate encapsulated hepatocyte spheroids. Spheroids-containing alginate beads were prepared in a size-controlled manner (700-800 μm in diameter) and exhibited the potentiality to sustain hepatocyte activities even in absence of non-parenchymal cells. Though, the inclusion of these latter determined enhancement of detoxification activity (ammonia removal and consequent urea and glutamine secretion) and slight improvement of synthetic functions (albumin and urea production) that was likely associated with a superior expression of connexin 32 and 43, responsible for homo- and heterotypic interactions but also for apical polarization of the HEPs. Fibronectin deposition may have also contributed to sustain the hepatic functions in these culture conditions. By contrast, non-parenchymal cells did not beneficially affect hepatocyte phase I/phase II biotransformation activities.

1. INTRODUCTION

Cell encapsulation, originally developed as promising therapeutic approach in tissue transplantation [1-3], has gained particular attention in disparate tissue engineering applications over the past fifty years [2]. In principle, cells are enclosed within a, generally polymeric, membrane which provides cell defense against destroying insults by high molecular weight molecules (antibodies and other immunologic moieties) while allowing the entry of nutrients and oxygen, indispensable for cell survive, and the exit of cellular products [4]. In addition, the three-dimensional (3D) structure of these membranes has the potentiality to re-establish a suitable *in vivo*-like microenvironment [5] supporting cellular metabolism, proliferation, differentiation and cellular morphogenesis [1]. Besides the beneficial isolation of a cell population from an outside environment, encapsulation membranes provide the contained bioactive materials with protection from mechanical strengths [6].

Hydrogels are attractive biomaterials largely employed as scaffolds for tissue engineering and, additionally, for cell encapsulation in 3D configurations [2]. Hydrogels derived from natural polymers mimic many features of extracellular matrix (ECM) and, consequently, are able to drive several cellular processes [7, 8]. Alginate has been widely utilized to prepare naturally forming and biodegradable hydrogels [2]. It is a linear polysaccharide of (1-4)-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers derived from brown seaweed [9]. Depending on the seaweed characteristics, the alginate polymer presents distinct distributions of the M and G monomers, organized in repeating or alternating blocks [9]. These different arrangements imply changes in diverse properties of the alginate polymer. Alginate gels are obtained by means of binds between divalent cations (Ca^{2+} , Ba^{2+} , or Sr^{2+}) and G-blocks which lead to the establishment of ionic bridges between different polymer chains [8, 10]. Alginate has been considered to be an adequate candidate for cell encapsulation due to its mild gelling reaction in the presence of calcium chloride, but also thanks to its properties of biocompatibility and non-toxicity [11].

Alginate-based-encapsulation has also found ample application in the liver tissue engineering field, more exactly implicated in the definition of therapeutic treatments for bridging acute liver failure patients to transplantation, such as intrahepatic hepatocyte transplantation [12, 13] and extracorporeal bioartificial liver (BAL) devices [14-17]. The main limiting factor to the development of these solutions is associated with the scant availability of hepatocytes (HEPs) as well as the inability of these cells to maintain their peculiar characteristics and functions for longer time spans [18, 19]. Microencapsulation of HEPs in alginate beads allowed cells retaining functionality and viability [14, 20] for extended *in vitro* culture periods compared to conventional monolayer culture. Beside the effective protection of the HEPs from external immune-system attacks [15], 3D matrices were ascribed to be responsible for the preservation of HEP polarity [21] that is unlikely maintained in standard two-dimensional (2D) culture. Alginate encapsulation of HEP spheroids, in place of single cells, provided an evident breakthrough in the domain. Several groups demonstrated that the entrapment of HEP spheroids in alginate beads could represent a promising strategy to enhance the preservation of hepatic functionalities *in vitro* [16, 22-27]. The added value of this culture condition was likely the setting-up of improved cell-cell interactions between HEPs in the spheroidal organization emulating the *in vivo* cellular structure [16, 28-30]. Few other studies [24, 31, 32] showed that liver-specific functions could be further stabilized over time by including the non-parenchymal cellular component in the HEP spheroids entrapped in alginate beads. The presence of non-parenchymal cells (NPCs) elevated the complexity of the system and provided HEPs with cues of the native hepatic microenvironment by means of the establishment of homo- and heterotypic interactions as well as the secretion of soluble signaling molecules or insoluble extracellular matrix (ECM) components [33, 34]. As consequence, more physiological events were triggered in a more *in vivo*-like situation. Regardless, hepatic co-culture approach has been scarcely investigated in the context of the encapsulation. Most of the available alginate-encapsulated spheroidal co-cultures did not satisfactorily mirror the intricacy of the liver cellular component. Though, No et al. [31] reported the construction of complex micro liver tissue constructs encapsulated in collagen-alginate composites which included hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs), in addition to the HEPs, for the purpose of mimicking the hepatic microarchitecture and improving HEP functions and survival *in vitro*. Despite the elevated degree of cellular complicatedness of this model, other important cues may be missing and related to the absence of Kupffer cells (KCs) that, together with LSECs and HSCs, primarily cooperate with the HEPs for the performance of the *in vivo* liver duties [35]. Current co-culture models also seem to overlook the involvement of the liver in many homeostatic functions and the importance of re-establishing similar activities in *in vitro* conditions. Consequently, most of the co-culture systems restricted their scrutiny to well-characterized synthetic functions, such as albumin and urea production, and cytochrome P-450 enzymes biotransformation activity [36].

In this study, we propose to apply the model defined in our laboratory (see previous chapters), constituted of HEPs, HSCs, LSECs, and KCs tightly packed in a spheroidal configuration according to pre-established cellular proportions, and encapsulate it in alginate beads. The objective, here, was to evaluate the reliability of this system in emulating the *in vivo* wide set of liver activities and speculate about its suitability for actual usages, including intrahepatic hepatocyte transplantation and BAL devices. To this end, disparate hepatic-specific functions were sifted through in the alginate-microencapsulated spheroidal multi-cultured model and compared to those of spheroids made of only HEPs and equally encapsulated in alginate microbeads.

2. MATERIALS AND METHODS

2.1. HEPATIC CELL ISOLATION

Hepatic parenchymal and non-parenchymal cells were isolated from male Sprague-Dawley rats (Janvier Labs, France) weighting 200-300 g by the two-step method of Seglen [37]. Sorting of the different cell populations was achieved by combining previous procedures [38, 39]. Briefly, the resulting digested liver cell suspension was centrifuged (50 g, 5 min, thrice). The resulting pellets were mixed and suspended in an Easycoll separating solution (Biochrom AG – Merck Millipore) prepared in Dulbecco's Modified Eagle Medium (DMEM, Gibco – Life Technologies) with final density of 36% and centrifuged (168 g, 20 min). The derived HEP-containing pellet was washed in DMEM (100 g, 5 min) and retained. The initial saved supernatants were split in two portions to isolate either HSCs or a fraction constituted of KCs and LSECs. To obtain HSCs, supernatants were first washed (50 g, 5 min) in order to discard the HEP-containing pellets. HEP-free supernatants were centrifuged twice (200g, 10 min) and the resulting HSCs-containing pellets were retained. The other initial supernatant portion was centrifuged (1350 g, 10 min) and the resulting pellets were mixed and loaded on a density gradient Easycoll separating solution (25-50%), next centrifuged (1350 g, 30 min). Only cells at the interface between the two density cushions were retained, suspended in DMEM and centrifuged (1350 g, 10 min). Final pellet contained KCs and LSECs (termed LSEC/KC-fraction). The entire procedure was carried out at 4°C. Cell viability was assessed by trypan-blue exclusion test on all isolated cell fractions.

2.2. HEPATIC CELL CULTURE FOR SPHEROIDS FORMATION

Isolated hepatic fractions were suspended in hepatic culture medium (HCM™ Hepatocyte BulletKit, Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco – Life Technologies). Suspended cells were inoculated into glass petri dishes (\varnothing x h = 60 x 20 mm) coated with Sigmacote® (Sigma-Aldrich) as following described. Multi-culture condition included HEPs and the different isolated NPC fractions in a ratio of 1 HEP and 2 NPCs (this latter containing 20% of HSC- and 80% of LSEC/KC-fraction). Specifically, 1×10^6 HEPs, 0.4×10^6 HSCs, and 1.6×10^6 LSEC/KC-fraction were seeded in 5 mL of media per petri dish. A mono-culture condition, constituted of only (1×10^6) HEPs, was used as control. Dishes were subject to continuous orbital agitation at 70 rpm with oscillation amplitude of 16 mm (SSL1 orbital shaker, Stuart) in a humidified environment at 37°C and 5% CO₂.

2.3. SPHEROIDS MICROENCAPSULATION IN ALGINATE BEADS

Formed spheroids were harvested from both multi- (hetero-spheroids) and mono- (homo-spheroids) culture conditions and maintained separately. After a wash in warm DMEM, spheroids were encapsulated in 1.5 % (w/v) of filtered alginate (MANUCOL® LKX 50DR, FMC BioPolymer) prepared in 0.9 % (w/v) NaCl solution. For both conditions, the spheroid-alginate mixture was prepared at a final concentration of 2×10^6 seeded HEPs/ml alginate. Microencapsulation was performed in an air-jet droplet generator system and alginate cross-linkage was accomplished in a CaCl₂ (120 mM) bath (pH 7.6) (**Figure 1.B**). Spheroids-containing alginate beads were inoculated in glass petri dish containing free-serum HCM. Culture was maintained for 7 days after encapsulation (day 3+7) under orbital agitation. Medium was changed 24 hours after encapsulation and, then, every three days. In correspondence to these culture days, functional characterization was carried out. A culture planning is depicted in **Figure 1.A**.

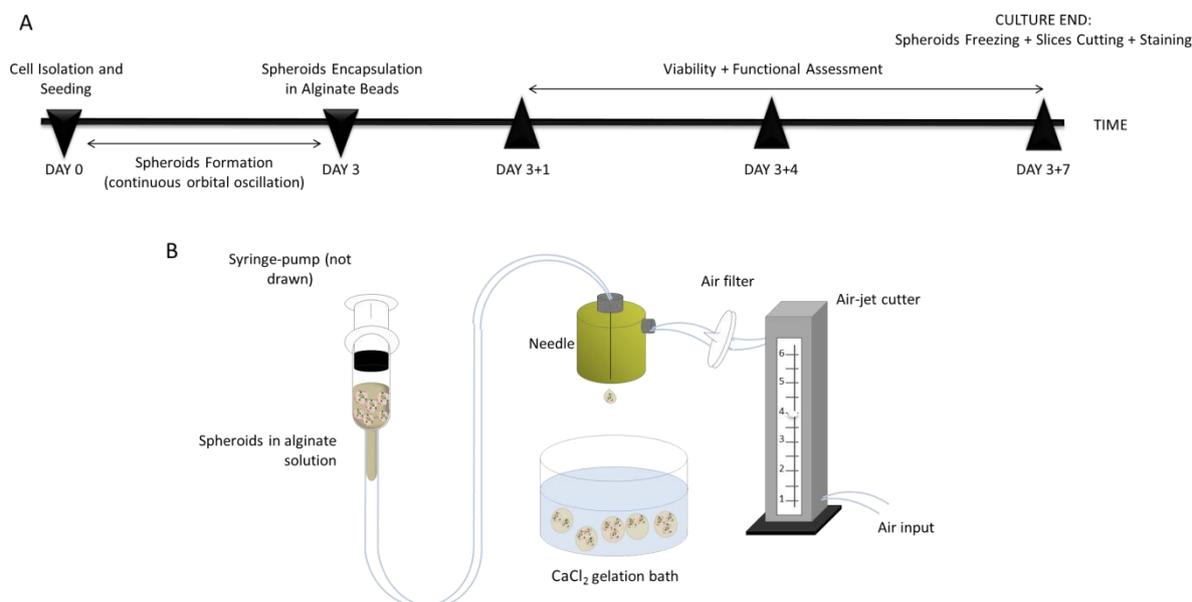


Figure 1. (A) Experimental time-planning and (B) schematic representation of the air-jet system used for spheroid microencapsulation in alginate beads.

2.4. CELL VIABILITY

Spheroids-containing beads were incubated in a solution of propidium iodide (10 $\mu\text{g/ml}$) and of Hoechst 33342 (10 $\mu\text{g/ml}$) in Williams' medium E for 30 min at 37°C under gentle orbital oscillation. Samples were washed in warm Williams' medium E and observed under epifluorescence microscope (Leica DMI 6000B, 20x/0.40). Used Williams' medium E (WE) was devoid of L-glutamine and red phenol (PAN Biotech).

2.5. ALBUMIN AND UREA PRODUCTION

Spent medium was collected from both conditions and stored at -20°C. Albumin synthesis was quantified by ELISA test (Rat Albumin ELISA kit, Bethyl Laboratories, Inc.), whereas urea secretion was determined by colorimetric assay (QuantiChrom™ Urea Assay Kit, BioAssay Systems). Analyses were performed in conformity with the manufacturer's instructions.

2.6. AMMONIA DETOXIFICATION

Microencapsulated spheroids were incubated in 1 mM NH_4Cl in WE for 2 hours under orbital agitation at 37°C. Supernatant was recuperated and stored at -20°C. Ammonia concentration was automatically measured with Konelab 20 analyzer (Thermo Fisher Scientific) by using enzymatic UV method (Randox). Assay was carried out according to the manufacturer's instructions.

2.7. GLUTAMINE SECRETION

Spent media, collected from microencapsulated spheroid cultures in 1 mM NH_4Cl in WE, was used to measure the levels of produced glutamine. Assay was carried out according to the manufacturer's instructions (Sigma-Aldrich).

2.8. URIDINE DIPHOSPHATE GLUCURONOLTRANSFERASE (UGT) ACTIVITY

UGT activity was evaluated in accordance with previous defined procedure [40, 41]. Alginate-microencapsulated spheroids were washed in WE, then incubated in 100 μ M of 4-Methylumbelliferone (4-MU), prepared in WE, for 2 hours under orbital agitation at 37°C. Supernatant was saved and used to measure the fluorescence (excitation wavelength of 320 nm and emission wavelength of 450 nm) by means of a fluorescence microplate reader (Spectafluor Plus, TECAN). The 4-MU remaining concentration was deduced from the fluorescence measurements based on a linear standard curve generated in WE.

2.9. ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY

EROD activity was estimated in conformity with previous defined method [42]. Alginate-microencapsulated spheroids were washed in WE, then incubated in ethoxyresorufin (10 μ M), prepared in WE, for 2 hours under orbital agitation at 37°C. The substrate included salicylamide (3mM) in order to inhibit phase II enzymes. Supernatant was retained and used to measure the fluorescence (excitation wavelength of 535 nm and emission wavelength of 595 nm) by means of a fluorescence microplate reader (Spectafluor Plus, TECAN). Resorufin formation was deduced from the fluorescence measurements based on a linear standard curve generated with a solution (ethoxyresorufin in WE) containing exogenous resorufin (10 μ M).

2.10. IMMUNOCYTOCHEMISTRY OF ENCAPSULATED SPHEROIDS

Microencapsulated spheroids were harvested, washed in WE and transfer in cryomolds containing embedding solution (Tissue-Tek O.C.T. Compound, Sakura Finetek). Embedded samples were acclimated for few minutes at 4°C, then frozen in liquid nitrogen and stored at -80°C. Samples were cryocutting at -32°C with 10 μ m section thickness and affixed to charged slides (Superfrost/Plus, Fisher Scientific). Samples sections were fixed in acetone, blocked in 3 % (w/v) bovine serum albumin (30 min), and incubated in primary antibody (1:50) for 1 hour. After abundant washes, samples were incubated in secondary antibody (1:150) for 1 hour in the dark and washed. Cell nuclei were stained with Hoechst for 2 min in the dark and mounted. Images were acquired under confocal microscope (Zeiss LSM 710, 63x/1.40 Oil DIC). Used primary antibodies are listed in **Table 1**, whereas secondary antibodies (Donkey anti-mouse Alexa Fluor® 488, Donkey anti-goat Alexa Fluor® 594, and Donkey anti-rabbit Alexa Fluor® 594) were purchased from Life Technologies.

Table 1. Primary antibodies used for encapsulated spheroids immunostaining.

Staining for...	Primary antibody
Extracellular matrix	Fibronectin ⁽¹⁾
	Collagen type I ⁽²⁾
Gap/tight junctions	Connexin 32 ⁽¹⁾
	Connexin 43 ⁽³⁾
	Zonula Occludens 1 ⁽¹⁾
	Claudin ⁽³⁾
Membrane transport proteins	Occludin ⁽¹⁾
	Mrp3 ⁽¹⁾
	Mrp2 ⁽¹⁾
	Bsep ⁽¹⁾
	Ntcp ⁽¹⁾
	Oatp ⁽¹⁾

Antibodies were purchased from Santa Cruz Biotechnology⁽¹⁾, Abcam⁽²⁾, and Invitrogen⁽³⁾.

2.11. ENCAPSULATED SPHEROID HISTOLOGY FOR PERIODIC ACID–SCHIFF (PAS) STAIN

Microencapsulated spheroids were harvested, rinsed in WE (twice), and fixed in Bouin solution. Samples were paraffin-embedded, sectioned at 5 μm , and affixed to charged slides (Superfrost/Plus, Fisher Scientific). Spheroid slides were stained in conformity with the PAS protocol provided with the kit (Sigma-Aldrich).

2.12. STATISTICAL ANALYSIS

Results were presented as mean \pm SD of three independent experiments (n=3). Statistical significance was determined using nonparametric Mann-Whitney test by means of Prism - GraphPad tool (considering two-tailed test and confidence interval of 95%). P-values <0.01 were considered to be significant.

3. RESULTS

3.1. ENCAPSULATED SPHEROIDS AND THEIR VIABILITY

Three days after seeding, cells were tightly packed exhibiting spheroidal shape. Homo- (derived from mono-culture of only HEPs) and hetero-spheroids (obtained from multi-culture of HEPs, HSCs, LSECs, and KCs) were highly viable (> 96 %) but morphological different (**Figure 2**), likely showing diverse compactness stages. Hetero-spheroids, in fact, presented a kind of external cellular crown surrounding the dense spheroid body. By contract, homo-spheroids manifested a more compact architecture. At this culture time, homo- and hetero-spheroids were encapsulated in filtered alginate; the resulting spheroids-containing alginate beads presented a diameter ranging from 700 to 800 μm including an average number of 7 spheroids per bead. Encapsulation did not affect spheroids viability which was well preserved in homo-spheroids (\geq 95 %), whereas a major mortality was detected in hetero-spheroids (viability of about 90-80 %) especially localized in correspondence to cells of the external cellular crown (data not shown). Along the culture time, alginate beads maintained their round shape and consistency, whilst encapsulated spheroids preserved elevated viability levels which, however, reduced on day 3+7 especially in hetero-spheroids (**Figure 2**).

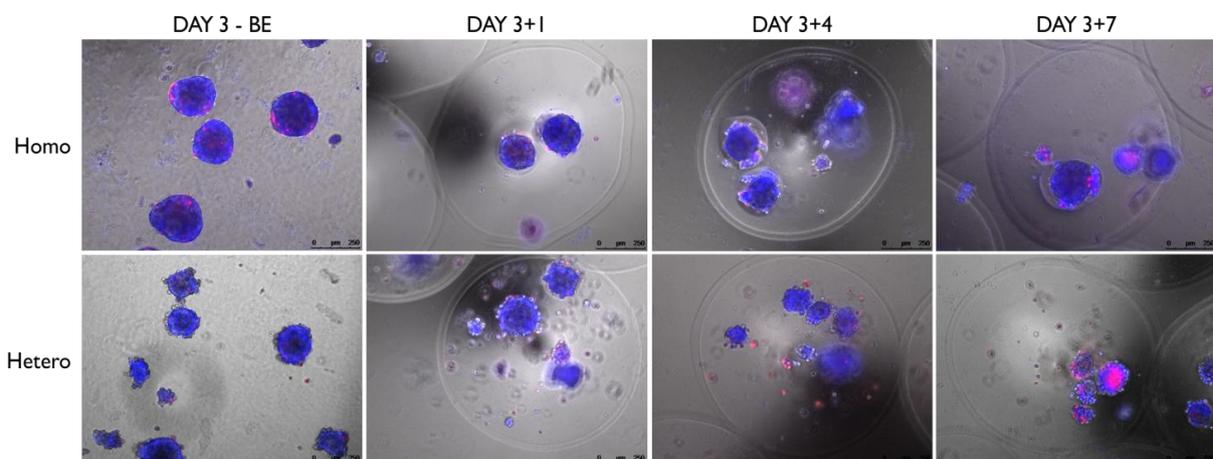


Figure 2. Cell viability in encapsulated homo- and hetero-spheroids over 7 post-encapsulation culture days. Viability was also assessed prior encapsulation (day 3 – BE). Alive cells are indicated in blue, while dead cells are showed in pink.

3.2. ALBUMIN AND UREA SECRETION

Albumin and urea productions were considered as indexes of metabolic and synthetic activities of the HEPs (**Figure 3**). In these circumstances, the presence of NPC populations in encapsulated hetero-spheroids was less effective on improving HEP synthetic functions. Albumin production underwent an important increase between day 3+1 and day 3+4 in both encapsulated homo- and hetero-spheroids (approximately 3- and 6-fold for the respective conditions); nevertheless, it decreased again between day 3+4 and day 3+7 of about 2-times in both conditions. By contrast, urea secretion remained almost constant along the time in both homo- and hetero-spheroids. However, encapsulated hetero-spheroids slightly enhanced HEP ability of producing albumin on day 3+4 as well as that of secreting urea on day 3+1 and day 3+7.

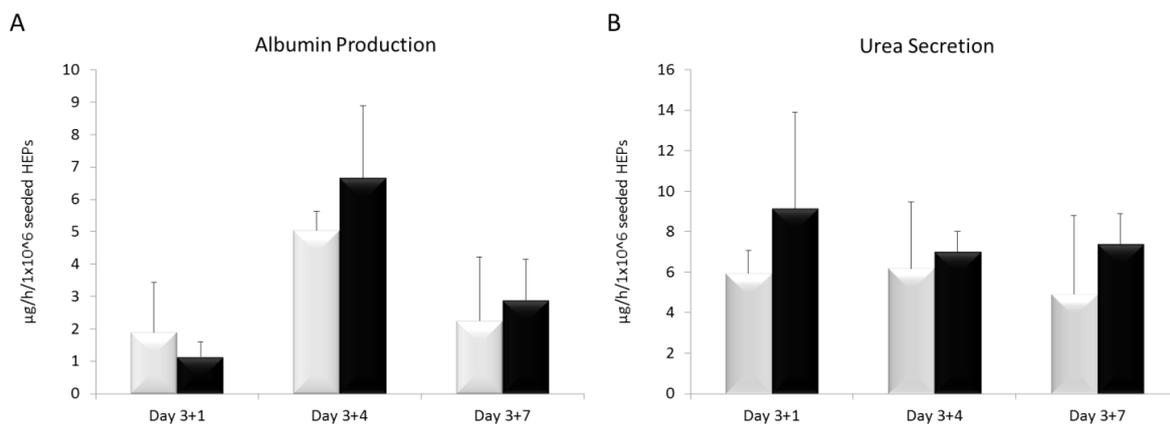


Figure 3. Synthetic hepatic functionalities, albumin (**A**) and urea (**B**) productions, of encapsulated homo- and hetero-spheroids. Grey bars represent homo-spheroids, black ones symbolize hetero-spheroids.

3.3. AMMONIA DETOXIFICATION, UREA AND GLUTAMINE SECRETION

Encapsulated homo- and hetero-spheroids retained their ability to detoxify ammonia (**Figure 4-A**). Although detoxification potentiality decreased with time (maximum ammonia elimination occurred on day 3+1), this capability was slightly better preserved by HEPs in encapsulated hetero-spheroids along the entire period. In these conditions, HEPs detoxified about 16 ± 4 % of the relative initial ammonia concentration (evaluated in respect to the ammonia concentration in beads empty of spheroids) in 2h on day 3+1. At this time-point, HEPs in hetero-spheroids eliminated 19 % more ammonia than HEPs in homo-spheroids. This difference was maintained on day 3+4 and became scarcely more important on day 3+7. In fact, HEPs in hetero-spheroids eliminated 34 % more ammonia than HEPs in homo-spheroids.

In accordance with this outcome, urea and glutamine were, generally, secreted in major quantity by HEPs in hetero-spheroids (**Figure 4-B, C**). However, this effect was relevant on day 3+7, when hetero-spheroids produced approximately two-fold more urea and glutamine than homo-spheroids.

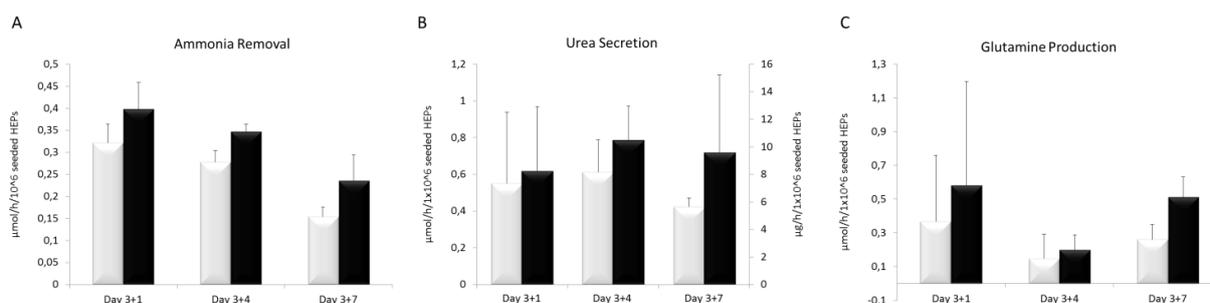


Figure 4. Detoxifying activity of encapsulated homo- and hetero-spheroids evaluated by ammonia removal (A) (in presence of NH_4Cl) and consequent transformation in urea (B) and glutamine (C). Grey bars represent homo-spheroids, black ones symbolize hetero-spheroids.

3.4. PHASE I AND PHASE II BIOTRANSFORMATION ACTIVITIES

The presence of non-parenchymal populations in spheroidal culture with HEPs in alginate beads did not beneficially affect biotransformation functionalities. In fact, hetero-spheroids manifested about equal (on day 3+1 and 3+4) or slightly reduced (on day 3+7) ability in metabolizing the 4-MU substrate (phase II) than homo-spheroids (Figure 5-A). Furthermore, hetero- and homo-spheroids also exhibited similar potentiality to convert ethoxyresorufin into resorufin (phase I) along the whole culture period (Figure 5-B).

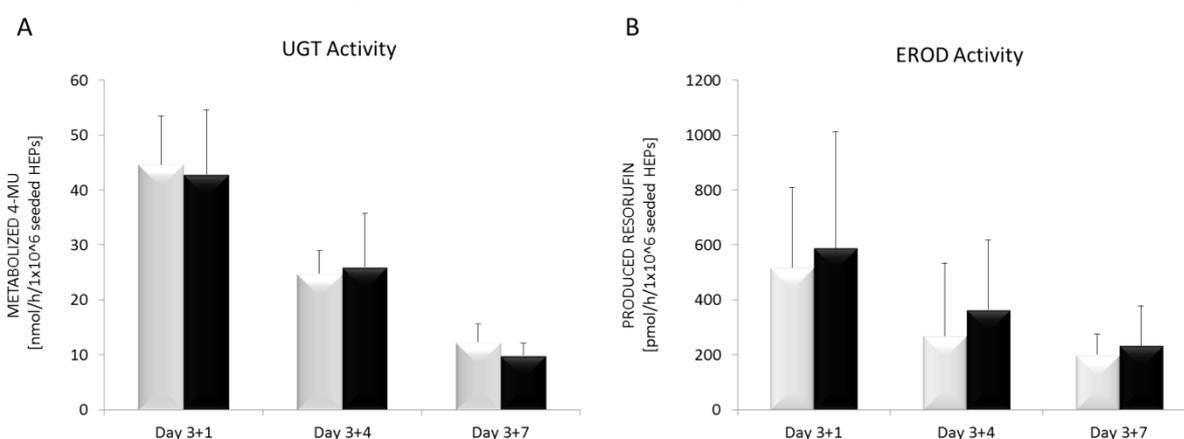


Figure 5. Phase II (UGT) (A) and phase I (EROD) (B) biotransformation activities of encapsulated homo- (grey bars) and hetero-spheroids (black bars).

3.5. CELL POLARIZATION (PHASE 0 AND III TRANSPORT)

Membrane transport proteins, identified by specific antibodies, resulted to be differently expressed in encapsulated homo- and hetero-spheroids (Figure 6). On the one hand, encapsulated homo- and hetero-spheroids generally manifested a similar expression level of phase 0 transporters, such as Ntcp and Oatp (Figure 6 A, B, C, D).

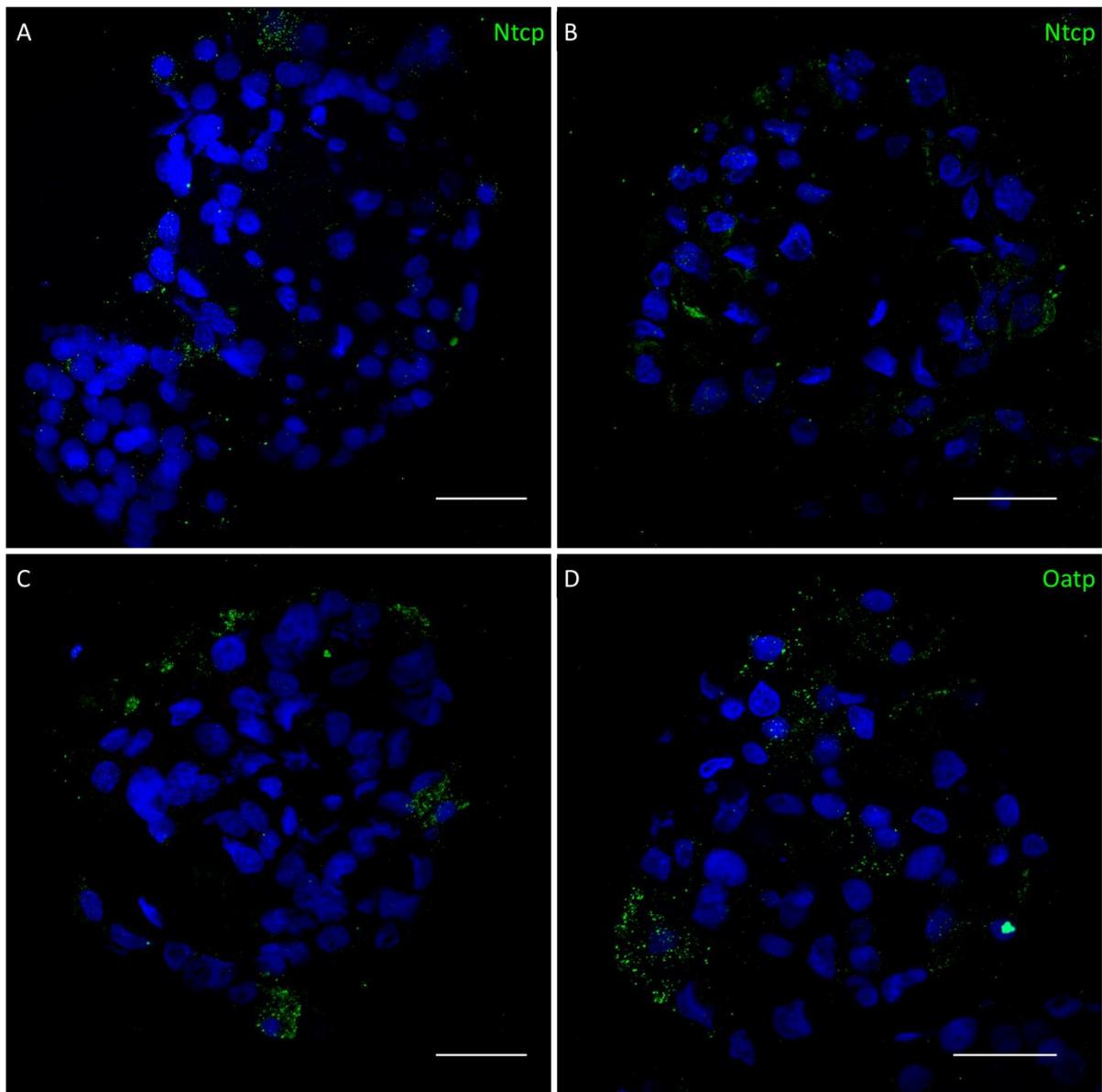


Figure 6. Hepatocyte polarization analyzed by immunostaining of membrane transport proteins (phase 0) in encapsulated hetero- (**A** and **C**) and homo-spheroids (**B** and **D**) at day 3+7. Scale bars equal to 50 μm .

On the other hand, the two spheroid categories showed dissimilarities in the expression of phase III transport proteins. Namely, canalicular Bsep was mainly present in hetero-spheroids (**Figure 6bis-G**), whereas, apical Mrp2 was uniquely expressed in homo- spheroids (**Figure 6bis-F**). Basolateral Mrp3-positive staining, instead, was detected in both hetero- and homo- spheroids (**Figure 6bis-E, F**), but the expression of this export protein (phase III) was diverse in the two conditions. In fact, hetero-spheroids presented either stippled (in major extent) or “circular”-perinuclear staining for Mrp3 protein; whilst, homo-spheroids especially exhibited “circular”-perinuclear staining for this transporter.

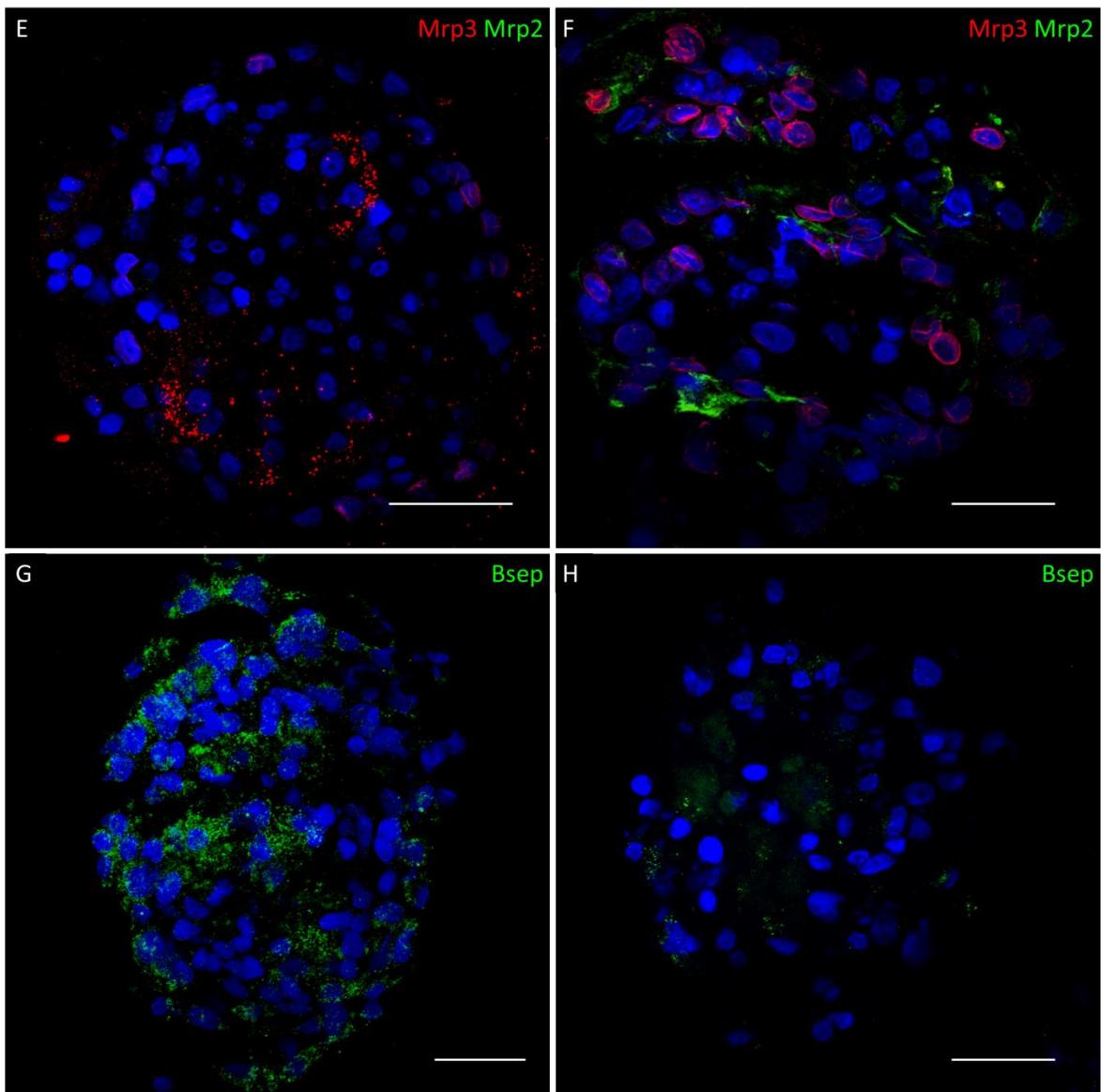


Figure 6 bis. Hepatocyte polarization analyzed by immunostaining of membrane transport proteins (phase III) in encapsulated hetero- (**E** and **G**) and homo-spheroids (**G** and **H**) at day 3+7. Scale bars equal to 50 μ m.

3.6. EXTRACELLULAR MATRIX COMPONENTS

Extracellular matrix components were detected in both encapsulated homo- and hetero-spheroids (**Figure 7**). Generally, a dense fibronectin mesh was identified in both spheroid conditions, while, collagen type I was absent or only present in the core of hetero-spheroids, but in sporadic and scarce amount.

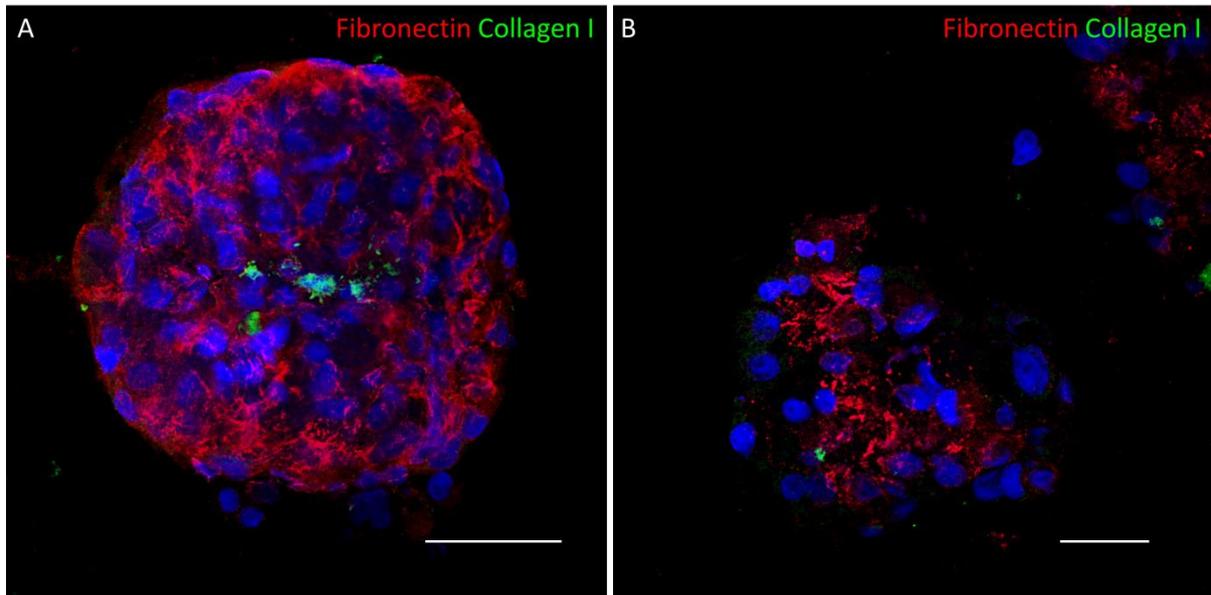


Figure 7. Extracellular matrix components (fibronectin and collagen type I) detected by immunostaining of encapsulated hetero- (**A**) and homo-spheroids (**B**) at day 3+7. Scale bars equal to 50 μm .

3.7. CELL (TIGHT AND GAP) JUNCTIONS

Tight (claudin, occludin, and zonula occludens (ZO)-1) and gap (connexins (cx) 32 and 43) junctions were analyzed by immunostaining for specific antibodies. Claudin-positive staining was observed neither in homo- nor in hetero-spheroids (data not shown). By contrast, occludin protein was sporadically found in both conditions, especially in hetero-spheroids, but the staining was unexpectedly localized in the cell nuclei (**Figure 8-C, D**). Similarly, both homo- and hetero-spheroids exhibited positive sites for ZO-1 which resulted to be stippled and, likely, distributed in equal manner in the two conditions (**Figure 8-E, F**). Cx 32 and 43 staining, instead, was clearly different between homo- and hetero-spheroids (**Figure 8-A, B**). These latter exhibited a pronounced number of sites expressing cx 32 and, mainly, cx 43. However, homo-spheroids also presented minor positive-staining for cx 43 and, especially for cx 32.

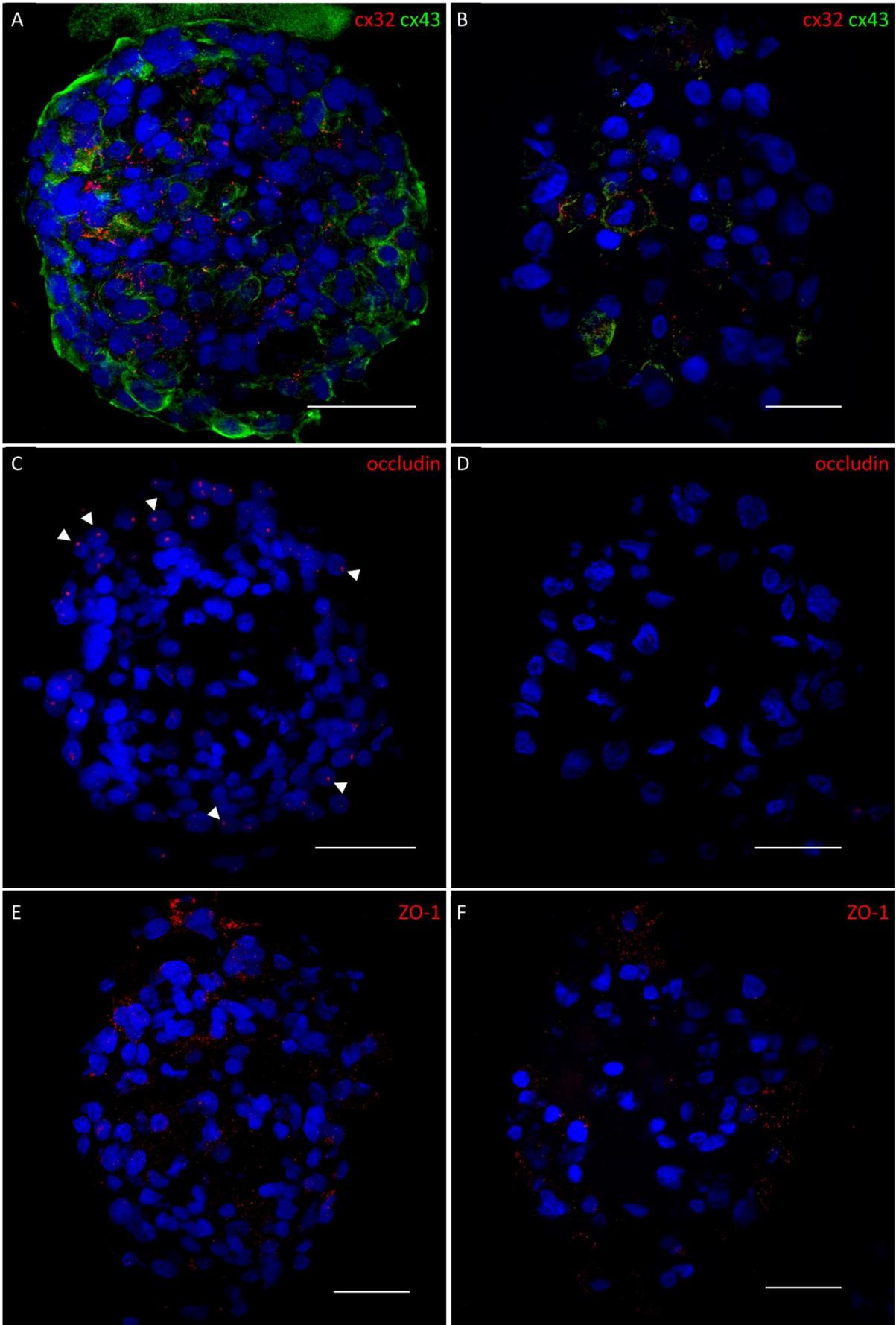


Figure 8. Gap (cx 32 and 43) and tight (occludin and ZO-1) junctions immunostained in encapsulated hetero- (A, C, E) and homo-spheroids (B, D, F) on day 3+7. White arrows (figure C) indicate nuclear occludin positive-staining sites. Scale bars equal to 50 μ m.

3.8. GLYCOGEN STORAGE

Glycogen was lightly stored in both homo- and hetero-spheroids (intense pink color); however, no difference was evidenced between the two conditions (**Figure 9**). In addition, apoptotic nuclei were identified in spheroid slides of both cases. This result was in line with the viability observation.

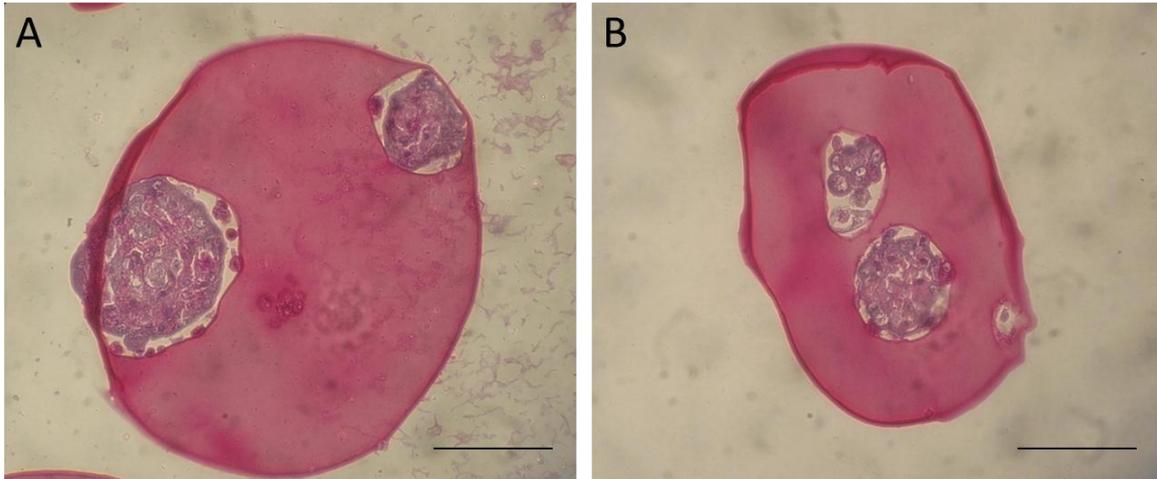


Figure 9. Glycogen storage in encapsulated hetero- (A) and homo-spheroids (B) on day 3+7. Scale bar equals 100 μm .

4. DISCUSSION AND CONCLUSIONS

Liver tissue engineering requires *in vitro* platforms that could adequately preserve the HEP functionalities for temporary therapeutic treatments, including cell transplantation and BAL devices. Encapsulation of single or aggregated HEPs has provided a significant contribution to the field. Preservation of encapsulated HEP functions has been documented in studies focused on transplanted animals [43] and BAL design [15]. These reports also highlighted the encapsulation matrix's essential role in immunoprotecting the bioactive cellular component. Alginate, which is a naturally occurring anionic polymer, has been largely utilized for HEP encapsulation [14-16, 44-48] due to its ability to undergo mild gelation in presence of divalent cations. Alginate gels' low protein absorption and high adjustable porosity as well as polymer characteristics (such as biocompatibility and low toxicity) and modest costs have really made this biomaterial a suitable candidate for *in vitro* encapsulation studies, readily translatable in *in vivo* applications [46, 49]. Alginate encapsulation of single HEPs, in fact, provided beneficial improvement in the expression of the differentiated hepatic phenotypes [50] and maintenance of liver-specific functions [20, 51, 52]. Therefore, the alginate-based-entrapment of HEP spheroids has been appointed as promising strategy in liver tissue engineering applications [16, 22-27] since *in vitro* hepatic functionalities were superiorly preserved thanks to the enhanced establishment of cell-to-cell contacts [23]. Nevertheless, this culture settings lack crucial elements of the intricate liver cellular composition and, consequently, those direct/indirect interplays which are fundamental in sustaining more physiological HEP responses. Accordingly, the inclusion of non-parenchymal populations in alginate-encapsulated HEP spheroids allowed further stabilization of liver-specific functions over time [24, 31, 32]. Yet, the co-culture approach has been scarcely considered in the context of the encapsulation.

The present study described a novel approach for liver tissue engineering applications consisting in the spheroidal culture of multiple hepatic cell populations in the 3D

environment of alginate beads. The system was prepared by pre-forming hetero-spheroids, composed of the three major non-parenchymal sinusoidal populations (LSECs, KCs, and HSCs) and HEPs, under orbital oscillation culture, as described in Chapter 4, and microencapsulating them alginate in a beads size-controlled manner. By means of this culture strategy, we intended to introduce further *in vivo*-like elements, such as a more adequate liver cellular complexity and a 3D arrangement for the establishment of tighter homo- and heterotypic interactions, in order to provide HEPs with more physiological cues, in addition to those favorably conferred by the alginate milieu, and to better support their viability and functions over time. By evaluating several specific HEP functionalities (albumin production, urea secretion, ammonia detoxification, glutamine secretion, and phase I/phase II biotransformation activities) and features (basolateral/apical polarization and cell junction expression), we speculated that 1) the presence of non-parenchymal populations differently modulated disparate HEP behaviors and 2) alginate microenvironment determined a good retention of HEP functionalities, already in absence of NPC component, making less evident the co-culture effect (as compared with previous results, Chapter 4).

If on the one hand, alginate beads may have enabled the encapsulated HEP spheroids to preserve their activities thanks to signals offered by the surrounding milieu resembling the extracellular matrices of the tissue [7, 53] and to mechanical protection from the dynamic orbital culture; on the other hand, the reduced co-culture impact may be ascribable to slight higher cell (likely NPCs) mortality in the hetero-spheroids than in homo-spheroids that may be consequence of stress induced by the encapsulation procedure on peripheral and less compact cells of the hetero-spheroids. From day 3+7, in fact, encapsulated hetero-spheroids generally started to manifest superior hepatic functions than homo-spheroids that may suggest a cell recovery from encapsulation. In these circumstances, NPCs exhibited distinct propensity in beneficially improving certain HEP activities rather than others.

Encapsulated homo-spheroids exhibited a good preservation of hepatic synthetic functions (albumin and urea secretion) which was, in addition, enhanced in comparison to that obtained in previous cultures of non-encapsulated homo-spheroids (see chapter 4, paragraph 3.5). This outcome is in line with previous studies that demonstrated improvement of, more in general, liver-specific functions in encapsulated homo-spheroids [12, 54] in comparison to non-encapsulated ones [16]. Encapsulated hetero-spheroids manifested additional slight enhancement of these activities compared to homo-spheroids in alginate beads; this may be due to the improved establishment not only of homotypic contacts between HEPs (connexin 32) but also of heterotypic interactions between NPC types (connexin 43) [33, 34, 55]. Cx32 is the major gap junction protein expressed by HEPs, but not by hepatic NPCs, and localized between adjacent cells [56]. Its expression is considered to induce the formation of tight junctions and, consequently, to influence HEP polarity [57-59]. In accordance with that, the superior manifestation of cx32 gap-junctions in the encapsulated hetero-spheroids may be also responsible for their better expression of the canalicular bile salt export pump (Bsep), in turn regulated through the expression of tight-junctions (zonula occludens (ZO)-1). ZO-1, which is primarily implicated – together with other tight junctions (occludin) – in the formation of hepatic polarity in the apical pole of the HEPs by sealing neighboring cells and avoiding intermembrane diffusions [60], was often localized in spheroid spots that also exhibited Bsep staining (as observed in both conditions). Especially in the hetero-spheroids, Bsep-positive staining covered larger areas than ZO-1 and appeared to be also situated inside the cells. As previously reported [61], such intracellular Bsep immunostaining may correspond to transporter-containing vesicles. However, the absence of the canalicular export pump multidrug resistance-associated protein 2 (Mrp2) in

the encapsulated hetero-spheroids resulted in contrast with the presented explanations. Mrp2 was only observed in encapsulated homo-spheroids.

Ammonia detoxification is a key function that requires to be replaced in liver failure patients. In these latter, in fact, the functionality is impaired and causes nitrogen imbalance in the body, that is, ammonia content increase in the brain and consequent cerebral disorders (including hepatic encephalopathy) [62, 63]. Healthy liver possesses two systems responsible for the removal of ammonia, the urea cycle and the enzyme glutamine synthetase, which are controlled by zonation [64, 65]. Hence, periportal HEPs convert ammonia into urea with low affinity but high capacity approach, whilst, perivenous HEPs convert ammonia into glutamine with high affinity but low capacity for ammonia [63, 65, 66]. In this manner, ammonia that slips away from the urea cycle conversion is then transformed in glutamine [64]. Thus, beyond the preservation of the ammonia detoxification ability, we also evaluated whether HEPs, independently from their zoned origin, maintained the two ammonia detoxification systems. The presence of NPC populations positively impacted on the ammonia detoxification capability of the HEPs in encapsulated hetero-spheroids. Consequent urea and glutamine secretion was higher in encapsulated hetero- than homo-spheroids; such productions were also more elevated than those measured in basal conditions (in absence of NH_4Cl). The incremental glutamine secretion in hetero-spheroid condition may be partially due to the contribution of KCs, which also expressed small amounts of glutamine synthetase [67].

On the contrary, NPCs presence did not beneficially affect phase I/phase II biotransformation activities of the HEPs in the encapsulated hetero-spheroids. Though, the resulting effects may underlie normal cell responses to drug presence. KCs, for instance, may be responsible for the release of pro-inflammatory cytokines (including tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6) that, in turn, may have induced downregulation of xenobiotic metabolizing enzymes (i.e. cytochrome P-450 (CYP) and UDP glucuronosyl transferase (UDPGT)) [68-72]. Although these effects were generally evaluated in *in vitro* inflammatory-induced settings (e.g. under stimulation with lipopolysaccharide (LPS)), there is evidence that KCs strongly suppressed the activity of at least CYP3A and UDPGT even in the absence of LPS stimulation [70]. However, the ineffective role of the NPC populations appeared less accentuated in the contest of the encapsulation (compared with analogous spheroid conditions devoid of alginate beads – Chapter 4) and differences between encapsulated homo- and hetero-spheroids became less remarkable (especially regarding the phase I activity). Alginate beads may play a role in this situation by protecting cells from the surrounding environment and reducing their direct drastic exposure to the drug, while allowing HEPs to better perform biotransformation in the encapsulated hetero-spheroids. However, this aspect requires further examination.

Similarly, any difference was found in the storage of the glycogen between encapsulated homo- and hetero-spheroids. This fact may be due to a loss of cell functionalities caused by an increase of mortality (for comparison, see Chapter 4). Therefore, such aspect remains to be further investigated at different other time-points.

In addition to a possible role of the alginate, the sustainment of the hepatic specific functions may be also associated to the intrinsic production of ECM components by the employed cells. In fact, HEP-ECM interactions have been considered fundamental in preserving HEP functionalities [73, 74] likely because resembling the native liver tissue which includes collagen, fibronectin, and laminin in the perisinusoidal space [75, 76]. HEPs are the major producer of fibronectin [77] even though, under specific conditions, HSCs contribute to its secretion [78]. Accordingly, a dense fibronectin mesh was weaved through the spheroid body of both conditions; whereas, collagen type I, which is normally secreted by

HSCs [79] but overproduced in case of fibrosis, was scarcely present in the core of encapsulated hetero-spheroids.

In conclusion, the combination of alginate-based-encapsulation and spheroidal culture of multiple hepatic populations may provide a breakthrough in the development of liver tissue engineering applications. In these conditions, in fact, HEPs may be induced to re-establish their physiological functions and behaviors permitting them to trigger more tissue-like responses.

5. ACKNOWLEDGMENTS

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ANNEX I

Last section of this PhD research work focused on studying hepatic responses of the *spheroidal multi-cultured model* in the microenvironment of the fluidized bed bioreactor (designed in our laboratory).

For the purpose, a small-scale glass fluidized bed bioreactor was employed in the investigations (**Figure 1**). This choice was conformed to low cell availability (rat primary cells) which would not be adequate to fill up standard big volume bioreactors (500 mL – 1 L volume for 10^9 estimated hepatocyte number). The small-scale bioreactor (which is a glass column closed by hole-screw caps to its extremities and having the following characteristics: internal diameter = 1 cm, height = 12 cm, and volume = 7.5 mL) was connected to a peristaltic pump (on its one side) and to a filtered-gas-exchange reservoir (on its other side) (**Figure 1**). Connections were done by means of oxygen-permeable pharmed tubing and classical silicon tubes (Cole-Parmer Instrument Company) having mean internal diameter of 2.5 mm. The bioreactor was filled up with (1 mL) alginate beads containing spheroids (for a final density of 2×10^6 seeded hepatocytes per 1 mL alginate solution) suspended in culture medium (HCMTM Hepatocyte Medium + BulletKit, Lonza). 13 mL culture medium was considered to be the minimum volume requested to fill up the entire perfusing circuit. In this study, 15 mL medium per condition were used.

The general working principle was to provide adequate flow such that it could generate a suitable expansion of “rest” alginate beads and ensure both good mass transfer and reduced mechanical stress on cellular component. Namely, under the effect of the perfusing flow, an initial column of alginate beads in “rest” condition (exhibiting a bed height h_0) underwent a such expansion so that its final stable bed height (h_f) was proportional to its initial height ($h_f = 2h_0$). This expansion was maintained by adjusting the flow rate during the entire culture period (10 days). Flow was interrupted every other day for medium collection (during specific analyses) and medium-change. Low flow rates (ranging between 1.5–3 mL/min) were employed in these investigations and set by the calibrated peristaltic pumps. Here, 2–4 pumps were simultaneously used in each experiment (**Figure 2**) in order to independently control different examined cases (encapsulated homo- vs hetero-spheroids – see CHAPTER 3 for their definition – analyzed according to diverse functional tests).

Cell viability was daily observed under epifluorescence microscope after encapsulated spheroid incubation in IP/Hoechst solution (see CHAPTERS 3, 4, or 5 for technical details). Following hepatic functions were assessed: 1) synthetic functionalities (such as albumin and



Figure 8. Small-scale glass fluidized bed bioreactors and complementary circuit accessories (medium filtered-gas-exchange reservoirs, connection tubes, and peristaltic pumps, on the back sides).



Figure 9. Multiple bioreactor-circuit set for independent control of distinct examined culture cases.

urea production), 2) biotransformation potential (phase I (EROD test) and phase II (UGT) activities), and 3) detoxification ability (ammonia removal). Related tests have been extensively described in previous chapters (see CHAPTER 5). Unfortunately, experiments did not provide the expected results. In fact, fluidized-encapsulated-spheroids underwent an important rapid drop of their viability (**Figure 3**) which critically affected hepatic functionalities. Therefore, functional outcomes won't be presented in this section.

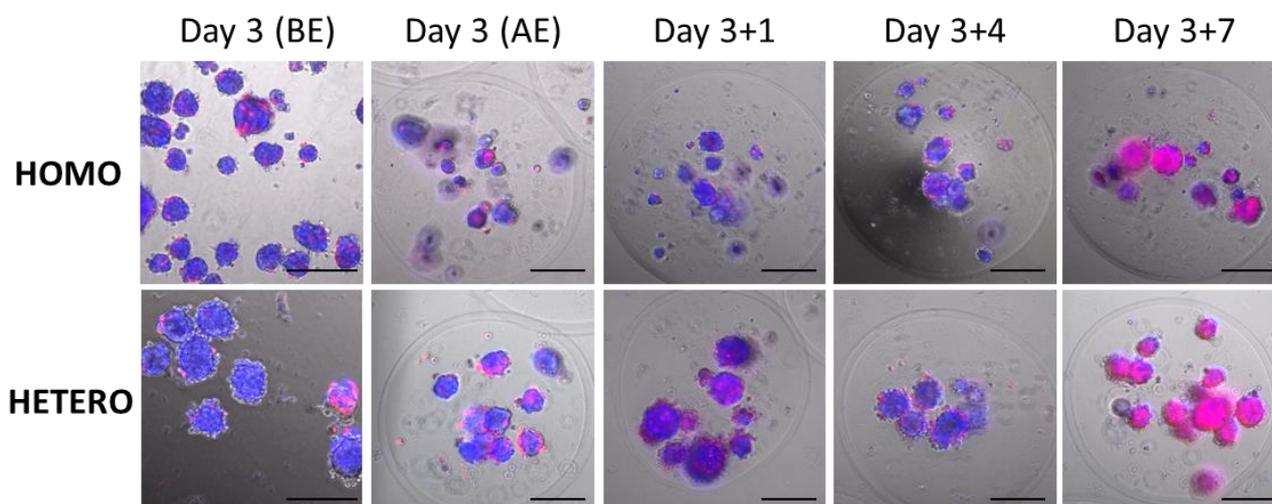


Figure 10. Cell viability in homo- and hetero-spheroids. Viability was evaluated before spheroid encapsulation (BE) as well as at the end of the procedure (AE). Moreover, viability was followed over the entire culture period (seven days after encapsulation, day 3+7). Pictures taken at some time-points are presented here. Blue color indicates alive cells, whereas, pink one represents dead cells. Scale bars equals 250 μ m.

By contrast, some suggestions will be detailed here in order to provide useful clues for future studies. Convincingly, cell mortality was determined by inappropriate fluidization of the spheroids-containing alginate beads within the bioreactors. In fact, beads seemed to remain tightly packed in the bottom part of the Sigmacote[®]-coated bioreactor. This behavior started to be visible few hours after fluidization beginning (run 1-2 hours after encapsulation) and became predominant after overnight fluidization culture. Though, it

disappeared after about two-three days of fluidization culture and beads were efficiently maintained at the optimal bed expansion height.

Although several modifications have been done to improve the culture conditions, situation did not change. Thereby, further studies should be carried out to understand causes and, consequently, adjust the culture parameters. Among different hypotheses, initial inappropriate fluidization may be due to oversynthesis of extracellular matrix material and/or fibrous proteins which may be result from cellular stress conditions (caused by subsequent processes of encapsulation and fluidization beginning). These components, released in the surrounding culture medium, may have compromised the correct fluidization of the alginate beads by generating a kind of “sticky-effect” between close beads that forced these latter to remain fixed at the bottom of the bioreactor. Logically, this fact reduced with time because a large amount of cells died and, therefore, release of these elements was reduced as fewer cells produced them.

Hence, future studies should first of all corroborate this hypothesis by performing specific staining for extracellular matrix material and/or fibrous proteins (for instance, fibrin) of spheroid-containing beads retained after overnight incubation on the first fluidization day. If outcomes will be as expected, then, culture approach should be modified. For example, incubation period between encapsulation and fluidization beginning should be prolonged so that cells could recovery from encapsulation and efficiently be fluidized when allocated in the bioreactor. Obviously, additional other causes may be responsible for inappropriate fluidization of spheroid- containing alginate beads. This possibility should be considered.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In this research work, a novel approach of co-culture, defined *spheroidal multi-culture model*, has been established for improving culture conditions of hepatocytes and preserving their functionalities in long spans.

The achievement of the *spheroidal multi-culture model* derived from a series of methods' setting-up and consequent outcomes achieved in several steps. The resulting conclusions and perspectives, for future investigations, are detailed below.

1. An important section of this work was devoted to the establishment of a cell isolation protocol. For practical reasons and, especially, to overcome the constraint of the human hepatic cells shortage, rat was selected as cell source. This choice may be argument of debates as isolated rat hepatic cells are, first of all, sane (compared with available human hepatocytes which frequently are isolated from discarded and pathological liver resections) and behave differently from human hepatic ones. Though, considering the challenging approach, this selection resulted to be suitable for the definition of a provisional model and for the determination of preliminary insights. Moreover, this choice was in line with previous similar investigations in the literature in which rat hepatic cells were broadly used. The isolation of hepatic cells possessed, in addition, disparate advantages that include expense reduction, in comparison with elevated cost of primary (especially human) cells, and usage of fresh cells, compared to cryopreserved ones.

This isolation procedure, based on previous similar techniques, represented, therefore, a reproducible inexpensive tool to obtain enriched fractions (of parenchymal and non-parenchymal hepatic cells) without involving complex laboratory equipment. Compared to previous techniques, this protocol allowed, in addition, obtaining multiple cell types from the same source and, likely, reducing interindividual variability in culture. Consequently, this also minimized the number of animals to sacrifice for the setting-up of a sole experiment. Considering the specific use of the isolated cell fractions in this study, the high enrichment level was considered sufficient but single cell purification was not optimized. Hence, the protocol should be refined if it will employ in other different contexts. In this regards, single cell sorting, from the enriched fractions, may be performed, for instance, by magnetic microbeads technology. Though, specific cellular membrane antibodies (necessary in this methodology) are still unavailable for the rat cell populations of interest; therefore, usage of unspecific proteins may entail presence of undesired cells in the final sorted fractions. Other techniques should be also taken in consideration.

2. The research core was developed in a consistent part of this PhD work that was organized in two consecutive phases.

In a first time, analysis of several culture parameters (four different media and cell ratios) provided optimal co-culture conditions for the culture of hepatocytes under spheroidal shape (evaluated in terms of viability support and improvement of albumin/urea production). On the one hand, media were formulated with different degree of enrichment complexity; namely, poorly-supplemented media were constituted of essential compounds, whereas, richly-supplemented media contained growth factors and other additives designed for specific support of cell differentiated states. On the other hand, cell ratios were established in order to define an optimal *in vitro* cell proportion which could mimic the complexity of the cellular composition of the liver. Therefore, a first ratio was defined to be reminiscent of the actual relative

cell proportions in the liver (60% HEPs and 40% NPCs). Additional ratios were also taken into consideration in order to adjust cell responses to *in vitro* conditions, considering that these latter do not necessarily correspond to *in vivo* cell behaviors. Results indicated that presence of non-parenchymal populations enhanced and preserved functional activities of the hepatocytes over time when cells, assembled under the shape of spheroids, were cultured in media suitable to maintain cells in a phenotypically active state. The functional improvement/maintenance was better in correspondence of higher non-parenchymal densities. However, the effect of the NPC-fractions was almost nil when the spheroids were cultured in a rich-supplemented medium. In these conditions, in fact, hepatocytes increased their secretion abilities both in presence and absence of non-parenchymal populations. It may seem logical to infer that medium composition was more important than co-culture. However, in these conditions, the co-culture effect may become relevant in the long-term (e.g. one month culture period). This aspect requires, therefore, more attention and must be a fundamental subject of future investigations.

Once optimal co-culture parameters (defining the spheroidal multi-cultured model) were set, additional peculiar hepatic aspects were examined in order to better characterize the system. Data demonstrated that non-parenchymal populations situated in specific locations within spheroids that were strongly reminiscent of their typical arrangement in the hepatic organ. Moreover, these cell types distinctly modulated different functions of the hepatocytes. In details, non-parenchymal cells tended to beneficially improve synthetic functions but not to have enhancing impact on biotransformation activities. However, this latter facet needs additional examination as it may result from normal events induced by the release of cytokines and modulators from non-parenchymal cells. Furthermore, it may be interesting i) to evaluate biotransformation cell responses under drug induction, and ii) explore more specific functional properties of the non-parenchymal populations. In addition, hepatocytes polarization, which was not greatly enhanced in presence of non-parenchymal cells in the present study, may be further analyzed and correlated to functional investigations in the future.

3. In the last section, spheroidal multi-culture model was used in the microenvironment of alginate beads. These latter are key components of the fluidized bed bioreactor as they provide immunological and mechanical protection to the bioactive component while allowing correct mass transfer (oxygen/nutrients entry and cellular products exit). Interestingly, functional assessment provided different outcomes in comparison with those obtained from culture of non-encapsulated spheroids (discussed above). In short, non-parenchymal cells, when encapsulated, exhibited reduced potentiality of preserving synthetic functions of the hepatocytes. This fact may be related to two distinct factors. On the one hand, alginate beads' microenvironment may have beneficially contributed to the retention of the hepatocytes' functionalities (already in absence of non-parenchymal populations), providing them with reminiscent signals of the native extracellular matrices as well as cell mechanical protection from the dynamic orbital culture. On the other hand, encapsulation procedure may have negatively impacted on "good health" of the non-parenchymal cells by reducing their viability and hepatic supporting capability. Non-parenchymal populations located on the spheroid surface were especially subject to damage. Thereby, potential solution may be the delay of the encapsulation step (4-7 days after seeding) to when external cells will be more integrated in the spheroid body in order to be less exposed to stress due to the procedure. This temporal change should be investigated.

By contrast, in confirmation to previous outcome, non-parenchymal cells did not enhance biotransformation activities of hepatocytes. As already stated, biotransformation functionalities should be further explored.

However, non-parenchymal populations had a role in improving the ammonia detoxification ability of the hepatocytes.

Additional modifications should be considered in the technique of encapsulation which may reduce cell damages and provide a real breakthrough in the performance of the model.

This model may represent, thereby, a promising tool to exploit, with the adequate adjustments, in the environment of the fluidized bed bioreactor (designed in our laboratory) to be applied in an extracorporeal circuit as bioartificial liver but also in diverse other areas of liver tissue engineering field (e.g. cell implantation, drug screening tests).

In future studies, the *spheroidal multi-culture model* should be used in the fluidized bed bioreactor. Experiments should be appropriately designed in this context (see Annex I).

Furthermore, although much remains to be refined, the *spheroidal multi-culture model* should be also transferred to different cell sources (e.g. human or porcine) in order to investigate its suitability in the development of bioartificial liver devices for clinical uses.

In this regards, some other facets (such as expenses and user practical aspects) should be taken in consideration. Even though spheroidal multi-culture model appeared to be beneficial for the functional preservation of hepatocytes, its scale-up into clinical practices will imply increase of cost associated, for instance, to purchase of higher volume of culture medium for cell culture and, consequent, building-up of the model. Moreover, model building-up (as broadly discussed above) requires usage of non-parenchymal fractions which need to be freshly isolated from liver. The isolation procedure is not easy to carry out and it is, especially, time-consuming (6-7 hours). Therefore, some cell banks should be created or, even better, banks of spheroidal multi-culture models should be produced in order to have available constructs always ready for any requirement. This strategy entails cryopreservation of cells or spheroidal multi-culture models. Cryopreservation procedures are well-known to be responsible for reduction of primary cell functions. In this context, thereby, another aspect that should be considered; that is, the ability of cells, cultured in the established model, to retain their properties even after thawing should be largely investigated. Several other facets should be analyzed beyond those mentioned in order to better evaluate the feasibility to introduce spheroidal multi-culture models in the actual practice.