# Final Publishable Summary Report

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# 1. Executive Summary

In HeMiBio, the aim was to create a bioreactor culture system of hepatocytes alone or in combination with the non-parenchymal fraction of the liver (hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs)) to allow repeated toxicity testing of cosmetics and chemicals for up to 2-3 weeks *in vitro*.

In the project we made the following advances beyond the state of the art:

1. CELLS: We characterized for the first time human primary **HSCs and LSECs** at the functional and transcriptional level; characterized transcriptomic as well as epigenomic processes that cause activation of HSCs, and developed methods to counteract this activation; while we demonstrated that LSECs very quickly de-differentiate in culture and developed medium that can delay this event for ± 2 passages; we developed **progressively improving methods to create hepatocytes from PSCs**, yielding cells that can be used to study toxicants. However, despite the significant improvement in hepatocyte progeny, the cells remain less mature than primary hepatocytes. Therefore, transcriptome, epigenome and metabolome studies were performed to understand hurdles in the differentiation process, insights which were and are continuously used to improve creation of mature hepatocytes. Likewise, cells with **HSC-like properties were created from hPSC**. Here too the progeny is not fully similar to quiescent HSCs from the liver. Transcriptome studies confirmed this and are now being used to further improve differentiation. A similar set of studies was also done to create **LSECs from either PSCs** or from **blood outgrowth endothelial cells**. In a third set of studies, the transiently immortalized UpCyte hepatocytes were fully characterized, and were shown to have functional properties that approach those of primary hepatocytes. These **UpCyte hepatocytes** (generated from 5 different donors) were suitable for toxicity testing.

SENSORS: In a second major set of studies, sensors to be incorporated in cells or in the bioreactor were created and tested. For **cellular sensors**, a genome edited set of stem cells was generated that now allows very fast recombination of any incoming cassette such as for instance the NFkB reporter. **For sensors within the bioreactor**, fully biocompatible microbeads equipped with an oxygen-sensitive, phosphorescent dye were incorporated within the bioreactor allowing real time detection of oxygen consumption. In addition, a novel ALT sensor was created, which can assess with high sensitivity the excretion of ALT from diseased liver cells. These sensors, combined with additional standard pH and glucose sensors were incorporated in two different switch boards/liquid handling units to allow intermittent sampling of culture fluid enabling assessment of the health of cells within the bio-reactor for protracted times.

BIOREACTORS: Three different bioreactor designs were generated: (1) **An antibody-based, microfluidic system**: capable of patterning any biotin-conjugated set of antibodies using streptavidin-based surface chemistry, allowing the generation of arbitrary cell patterns from heterogeneous mixtures in microfluidic devices. (2) **A flow-over bioreactor**: this stainless steel bioreactor protects hepatocytes from shear forces while creating stable oxygen and nutrient gradients mimicking the *in vivo* zonated liver. We demonstrated that HepG2/C3A cells could be maintained for over 28 days *in vitro*, while displaying over 98% viability and high expression of liver specific markers including CYP450 enzymes. (3) **A flow-through bioreactor**: although very challenging, significant progress was made towards producing a 3D COC-based bioreactor for liver cell culture, and most technological hurdles in producing prototype reactors were overcome. Further testing will be needed to ensure cell viability.

TOXICITY TESTING: The ultimate goal was to exploit the technologies, in toxicity studies. **UpCyte hepatocytes and PSC hepatocytes** were shown to be suitable for testing molecules shortlisted by the SEURAT-1 consortium on the gold compound list. A significant amount of work has also gone **to develop an *in vitro* model for liver fibrosis**, using co-cultures of HSCs and hepatocytes. These cocultures can identify fibrosis inducing drugs, as deposition of cross linked collagens can be detected following e.g. repeat dose exposure to methotrexate, a premier liver fibrosis inducing drug, among others.

*In conclusion:* HeMiBio has developed numerous tools towards the creation of innovative bioreactors, including cells, sensors and the reactors themselves to allow 2-3 week culture of hepatocytes with or without non-parenchymal cells to study the effect of drugs from the gold-compound list from SEURAT-1 and to for the first time allow assessment of liver fibrosis inducing drugs.

## 2. Summary description of project context and objectives

Refinement, Reduction and Replacement of the use of animals in toxicity tests is of particular importance for the implementation of relevant EU policies, such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation or the Cosmetics Directive (76/768/EEC). Although multiple projects had been funded by the EC aimed at decreasing the need for animals in toxicity testing before the start of the HeMiBio project, the assessment of toxic effects of chronic exposure still required and requires a relatively high consumption of animals. Moreover, aside from these ethical considerations, there was also a great need for suitable human cells to be used in toxicity testing, due to the often poor concordance seen between animal models and toxic effects in humans.

HeMiBio proposed to generate a **liver-simulating device** mimicking the **complex structure and function** of the human liver. The device should reproduce the interactions between **parenchymal** (hepatocytes) **and non-parenchymal** (hepatic stellate cells (HSC), and hepatic sinusoidal endothelial cells (HSEC) cells of the liver for **over 1 month *in vitro* in a high-throughput format**. Such a **He**patic **Mi**crofluidic **Bio**reactor should serve to test the effects of chronic exposure to chemicals, including cosmetic ingredients.

We postulated that to recreate a liver-simulating device suitable for long-term toxicity testing, (1) the cellular components of the liver need to be viable for extended periods of time (more than 1 month), with appropriate metabolic and transport function, and physiology that is comparable to the *in vivo* liver; (2) the device should allow fluid to flow over or even through the mixed cells that recreate a small liver section; (3) recreate the differences in function of hepatocytes and non-parenchymal liver cells depending on where they are localised near the artery or vein of in the liver; (4) assess the role of the non-parenchymal cells on the function and downstream toxicity of the hepatocytes, such as is the case in liver fibrosis. Such a device should be able to (iv) screen drug-drug interactions as well as long-term toxicity of chemical entities.

**WHAT WAS KNOWN AT THE START OF** HeMiBio**:**

It was/is believed that culture systems that incorporate hepatocytes as well as the non-parenchymal cellular components of the liver could be created to provide clinically relevant information on not only short-term but also mid/long-term drug clearance and drug toxicity of chemicals, i.e. over a period of at least one month, which can be used in the cosmetics industry. However, no reactor has yet been created that can indeed fulfil all criteria set forth above. Several culture methods have been evaluated, from simple cultures of hepatocytes in 2D- on extra-cellular matrix (ECM), to sandwich cultures where hepatocytes are cultured between two layers of ECM and 2D-flat membrane bioreactors, to co-cultures of different liver cell components in 2D-culture systems, and ultimately more complex 3D-culture and bioreactor systems, consisting of multiple or single compartments wherein the different liver cell components are co-cultured together. With increasing complexity, hepatocyte function is maintained better, whereas the less complex culture systems are more amenable to studying the mechanisms that control maintenance of cellular function.

These studies demonstrated that (1) co-culture of the different cellular compartments of the liver improves the long-term stability of hepatocytes as well as the non-parenchymal cells *in vitro*, which may ultimately lead to (2) the ability to use such devices for testing the effect of toxins directly on hepatocytes, or *via* their effects on the non-parenchymal component of the liver on the function and clearance ability of hepatocytes. Features of a liver-simulating device that play a role in the functionality of the device include (a) the matrix whereupon cells are maintained, (b) oxygenation, (c) shear flow, (d) transport phenomena, (e) the combination of cells included, (f) distance between the different cellular components, among others.

**THE UNDERLYING HYPOTHESIS**

The hypothesis for the successful creation of a 3D liver -simulating device suitable to test repeated toxicity was that

(1) **parenchymal (hepatocytes)** and **non-parenchymal cells (LSEC, HSC)** need to be combined.

(2) **cellular interactions between the different components are required** to maintain the functional, differentiated and quiescent state of both the parenchymal and non-parenchymal cell component.

(3) **matrix** whereupon cells are maintained, **oxygenation**, **nutrient transport** needs to be optimized to support long-term maintenance of parenchymal and non-parenchymal function

(4) the system needs to be built such that **repeated on-line assessment** of cellular integrity, as well as metabolic and transport function, and physiology of the different cellular components is possible.

**To achieve the creation of a bioreactor taking into account these hypotheses, the specific objectives were therefore:**

1. **Engineer the different cellular components** required for the development of a 3D-bioreactor **(WP1-2)** to:

(1) allow **specific and spatially defined enrichment** of the different cell components, using specific antibodies/ligands.

(2) allow **non-invasive detection by fluorescence read-outs** of the cellular state (

To accomplish this the following tools would have to be generated:

a. Use PSC-derived progeny (hepatocytes, LSEC or HSC)
b. We would also assess if cells other than pluripotent stem cells could be used to populate the liver device, including primary stellate cells, primary liver sinusoidal endothelial cells, or cells that have been transiently immortalized using the Medicyte (UpCyte) technology.
c. Use micropatterned cell-specific antibodies that would allow spatially specific immobilization of the different cell components in 2D-laminar flow in microfluidic devices and, if needed, also 3D-bioreactors.
d. We would knock in targeting constructs that code for fluorescent proteins and 3' from selected genes characteristically expressed in terminally differentiated cells, to assess *in vivo* and in real-time cell fate and allow reselection of cells from reactors.
e. We would knock in cell damage-specific expression cassettes (*e.g.* based on the activation of NF-κβ, …) in the *AAVS1* region, to assess *in vivo* and in real-time cell damage.

This should ultimately make it possible to determine which of the cell types in the liver-simulating device undergoes cell stress/death due to a given toxin.

*OBJECTIVE 1:* HeMiBio aimed to develop tools to engineer the cellular components for the bioreactor: to allow **specific and spatially defined** enrichment of the different cell components; to **non-invasively and in real-time assess** the **differentiation** state of the parenchymal and non-parenchymal cells as well as **cell damage**.

2. **Generate innovative sensing tools** that would **allow for non-invasive detection of the cell state/fate:** this would include biological fluorescent sensors for the dynamic readout of cell function and health (see objective 1, above). In addition, we would generate a combination of **electro-chemical sensors** to be embedded in the bioreactor to provide continuous measurement of **liver-specific function and cellular health**. These might include glucose, oxygen, potassium, ammonium, and ALT sensors. Such sensors should be able to dynamically assess the health/activation state of the different cellular compartments in a high-throughput format and provide critical information regarding long-term cellular function as well as repeated dose toxicity screening.

*OBJECTIVE 2:* HeMiBio aimed to incorporate **molecular sensors** to **dynamically measure cellular function and toxicity in a high-throughput format.** High-resolution fluorescent markers will be developed and integrated in a targeted fashion into the host cell genome to detect early inflammatory and pro-apoptotic effects (see also Objective 1). In addition, innovative electro-chemical sensors, such as ion-selective electrodes, would be integrated in the 3D-bioreactors to allow assessment of **liver function** (*e.g.* oxygen uptake, ammonium, and glucose concentrations), and also the continuous assessment of **cell integrity** (*e.g.* by measurement of potassium, and ALT release due to cell death).

3. As a rapid intermediary to the complex 3D-bioreactor, **2D-isolation-patterning bioreactors would be created** that allow for the efficient transition from a mixed iPSC culture to a spatially controlled organ-simulating device. In addition, a 2D flow over bioreactor would be created, wherein sensors developed are integrated in the bioreactor, providing critical insight into the evolving design of the 3D-bioreactor. In addition, this platform would also serve for the optimization of culture medium formulation for maintenance of long-term function.

*OBJECTIVE 3:* HeMiBio would develop a 2D-bioreactor for the efficient isolation of differentiated iPSC mixtures by trapping different cell types on micropatterned surfaces.

4. The **He**patic **Mi**crofluidic **Bio**reactor (HeMiBio) would be developed and enhanced with the development of integrated sensors and their characterization under microfluidics. Based on an established packed-bed bioreactor design previously shown to support the function of primary hepatocytes for over a week, HeMiBio would support physiological-level perfusion of self-assembled hepatic organoids in an individually addressable microfluidic array for high-throughput screening. Similar self-assembled hepatic organoids were shown to support liver-specific function of primary hepatocytes for over 40 days under static conditions. The device will be further fitted with our biological and chemo-electrical sensors that provide **dynamic high-throughput assessment of cellular function and health.**

*OBJECTIVE 4:* HeMiBio would generate a **liver-simulating device** mimicking the human liver, which reproduces the function of the **parenchymal** (hepatocytes) **and non-parenchymal** (HSC and HSEC) **liver** cells **over 1 month in culture**. This will be accomplished by combining engineered cells and the electrophysical sensors. The liver-simulating device created in HeMiBio will thus allow for the **dynamic monitoring of cellular function and health in a high-throughput format** under numerous conditions.

5. During the final years of HeMiBio, proof-of-concept studies would be performed to assess the effect of known chemical entities on the cellular components of the liver-simulating device. The choice of compounds to be tested would be adapted throughout the tenure of HeMiBio based on studies from the SEURAT-1 cluster. The HeMiBio platform was composed of not only hepatocytes but also non-parenchymal cells, and should therefore be very well suited to study toxicants that cause liver fibrosis, which is due to the activation of HSCs that form scars, and then lead to the death of hepatocytes.

*OBJECTIVE 5:* HeMiBio will provide proof-of-principle that a **liver-simulating device** can recreate the toxicity profile *in vitro* of toxins with a known *in vivo* toxicity profile **over a minimum of 1 month**, with specific emphasis on liver fibrosis

6. Throughout WPs 1, 3, 4 and 5, we planned to assess the phenotype of the different cellular components using a combination of **transcriptomics, epigenomics and metabolomics**. At all stages of reactor assembly, the **functional properties** of the different cell populations would be assessed using state-of-the-art functional assays. We hypothesized that **the phenotype of the parenchymal and non-parenchymal cell** components would be more similar to that of primary liver-derived cells when cells were cultured in **2D-cultures, compared with cells isolated from the liver/blood and cultured separately** or cells generated under the established iPSC-liver differentiation cultures; and that the phenotype of the two cell compartments of the liver **maintained in 3D-cultures would approach that of primary liver isolates even further**.

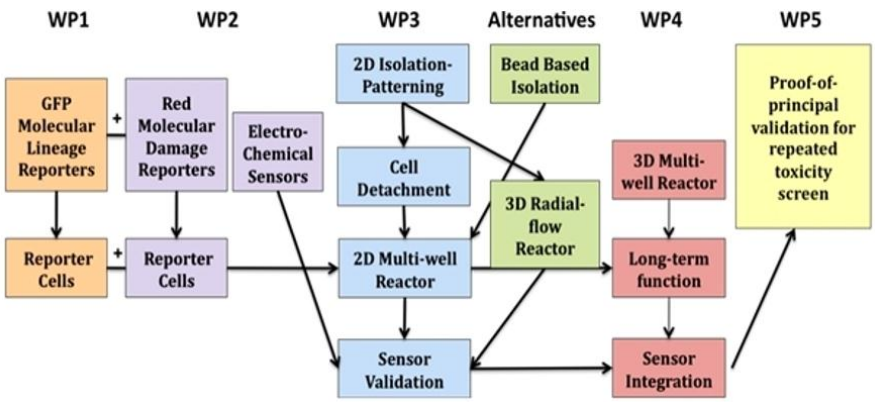
*OBJECTIVE 6:* HeMiBio planned to assess the **molecular, functional and metabolic phenotype of the hepatocellular, HSEC and HSC components at all stages of bioreactor development**, and compare this with that of cells isolated fresh from human livers.

## Overall strategy of the work plan

HeMiBio wished to create a prototype microfluidic device containing parenchymal (hepatocyte) and non-parenchymal (LSEC and HSC) liver cells for repeated toxicity testing of cosmetic ingredients and pharmaceuticals, to ultimately eliminate the need for laboratory animals in toxicology testing.

hiPSC, cultured under standard differentiation protocols, yielding immature hepatocytes and cells with some features of HSC and LSEC, would be used, as they can be generated from individuals with different metabolic and toxicity profiles, and expanded for more than 100 PDs, thus representing a steady source of cells for liver-simulating devices. As an alternative, we planned to test if primary hepatocytes, HSC and LSEC can be expanded using Medicyte's proprietary technique for 30-40 PDs, without losing important cellular functions. If the expansion procedure were to cause irreversible de-differentiation, Upcyte® hepatocytes, HSC and HSEC would still be very useful for the initial development of the bioreactors to be built in WP3 and WP4. In addition, we

would consider primary liver derived cells, such as stellate cells and endothelial cells, provided that they could be cultured for some time *in vitro*.




iPSC would be genome engineered to incorporate (1) selection cassettes (fluorophore) downstream of either a gene expressed specifically in mature hepatocytes, HSC or LSEC (**WP1**); (2) or cell damage-specific expression cassettes (p53, NF-κβ) incorporated, using Flp between FRT sites to be recombined in the AAVS1 region (**WP2**). This combination would allow precise detection of toxic effects on any of the three cell components to be incorporated in the device.

As a second means of following the health of the cells, we would create several electronic microsensors to be located in the direct vicinity of the cells to measure in real-time or at specific interrogation times (*e.g.* after a toxic insult) relevant parameters of the health of the cells (**WP2**). Microsensors would also be generated that can monitor cell culture conditions to aid in optimization of the culture medium (**WP2**).

In **WP3 and 4,** sequentially more complex bioreactors would be built to culture hepatocytes, HSC and HSEC for >28 days. We hypothesized that this would lead to further maturation of immature cells derived from iPSC AND assure their persistent differentiated and quiescent state for lengthy periods of time. In **WP3,** we would test if hepatocytes, HSEC and HSC can be captured from mixed iPSC cultures by microfluidic isolation on hepatocyte, HSEC and HSC-specific antibody-micropatterned surfaces. This would also allow testing if such micro-patterned co-cultures support differentiation and long-term maintenance of liver-specific functions. In a second step, we would also create a 2D flow over bioreactor, that could be used to test the effect of bioreactor mediated culture on maturation of iPSC derived cells, or initial studies of toxicants on hepatocytes, as well as testing of the sensors created in WP3.

The function of the different electronic microsensors from WP2 and the molecular sensors introduced in the cells in WP1/WP2, would be tested first in the 2D-bioreactors in WP3, and if validated subsequently incorporated in the 3D-bioreactors in WP4. In WP3 and WP4 we planned to also ensure that the molecular and electronic sensors provide the information aimed for, i.e. the continuous (intermittent) *in vivo* assessment of the differentiated state of the three cellular components, their overall state of health and ultimate function and survival of the liver-like tissue *in vitro.*

As the ultimate goal of HeMiBio was to use the to-be-created device as an alternative to rodent toxicology studies, it will be of the utmost importance to evaluate if the 3D-bioreactors reveal the toxicity expected from a number of prototypical hepatotoxic compounds known to trigger clear-cut liver injuries *in vivo*. As proof-of-concept, the 3D-bioartificial liver-device would be treated in **WP5** with cosmetic ingredients with suspected toxicity. A prerequisite to accomplish this critical task was the establishment of a set of function and toxicity screening assays as well as a list of test compounds, which will be done in close collaboration with investigators in call **HEALTH-2010-4.2.9-6**.

Throughout **WP1, 3, 4** and **5**, we planned to use classical "-omics" and functional studies to assure that the cellular components are liver-like, and to assess the effect of the toxic compounds on that state.. This was planned to also be done in collaboration with the team responsible for "-omics" for the consortium as a whole (Call **HEALTH-2010-4.2.9-5)**.

In **WP6** (Training and Education) we planned a series of education and training opportunities to train young scientists within HeMiBio and beyond, in the highly innovative and technically challenging concepts embedded in the proposal. Finally, we employed a professional management group to ensure the smooth and professional administration and implementation of the above strategy described in **WP7** (Management and Coordination).

# 3. Main S&T results

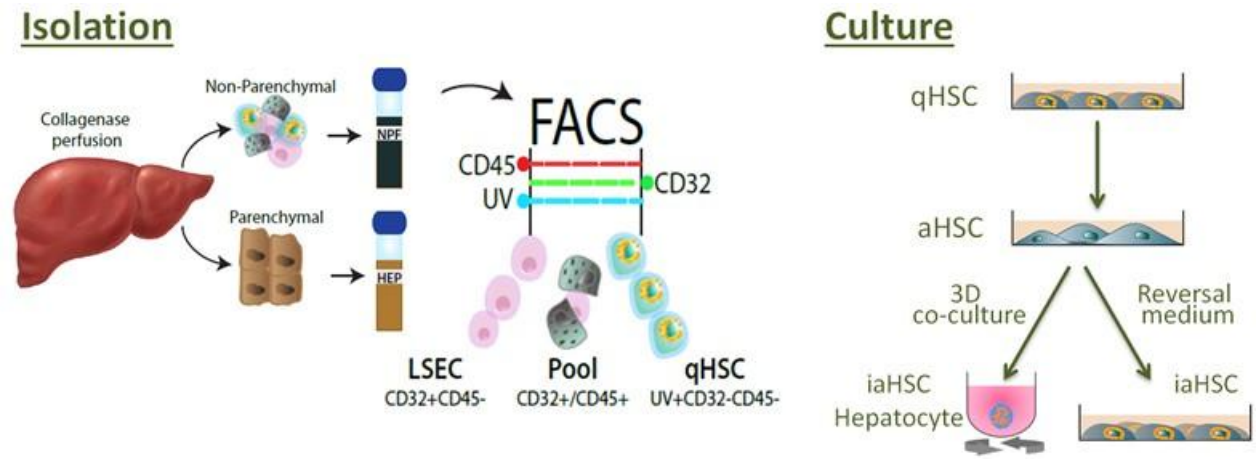
## 3.1. Cells to incorporate in the bioreactor

### 3.1.1. Primary liver cells

#### 3.1.1.1. Hepatic stellate cells

*i. Isolation and culture*

Hepatic stellate cells (HSCs) are the main effectors of liver fibrosis, independent of the etiology, they activate upon liver injury and inflammation and produce large amounts of extracellular matrix resulting in scar tissue formation and liver fibrosis. HSCs are routinely obtained from rodents by in si*tu* perfusion of their livers with solutions containing pronase and collagenase, followed by cell separation by density gradient centrifugation (e.g. 8% Nycodenz®) and typically yields relatively pure qHSC populations (90-97%). This procedure may be completed by fluorescence-activated cell sorting (FACS) where HSCs can be further purified (up to 99%) based on a positive selection for ultraviolet-positivity (retinyl esters autofluorescence at 328 nm). In HeMiBio we optimized this procedure for human donor livers, by using UV positivity combined with a negative selection for CD32 and CD45 in FACS-based isolations of the non-parenchymal fraction. We used this procedure to obtain highly pure HSCs for gene and miRNA expression as well as DNA methylation studies comparing freshly isolated quiescent "healthy" HSCs with fully activated "fibrotic" HSCs.




**Isolation and culture of human HSCs.** Human liver cells were isolated from healthy donors using a two-step perfusion technique. Parenchymal cells were removed by low-speed (50 g) centrifugation steps. Enriched populations of human qHSCs were sorted out through a negative selection for CD32 and CD45 expressing cells and a positive selection for ultraviolet positivity (retinyl esters auto-fluorescence at 328 nm), using a fluorescence activated cell sorter (FACS). qHSC activate when culture in regular mono-layer 2D culture conditions. Activated HSCs exposed to reversal medium or co-cultured in 3D with HepaRG cells regain a quiescent like phenotype here referred to as inactivated HSCs (iaHSCs).

Once HSCs are purified, culturing them on a stiff plastic surface leads to their transformation towards fibrogenic myofibroblasts. Rodent HSCs cultured on soft matrices or cultured in 3D spheroids show a lesser activation than cells cultured in traditional 2D cultures. During HeMiBio we developed culture conditions that can lead to a more quiescent phenotype of human HSCs: (i) by exposing 2D monocultures to a proprietary medium (Reversal medium) that can push the human HSCs towards an inactivated phenotype, a HSC cellular state that resembles quiescent HSCs but is more prone to activation, (ii) through co-culture of human HSCs with differentiated human HepaRG® cells (hepatocyte-like) in 3D organoids, HSCs obtain and maintain a more quiescent-like state which is characterized by low secretion and deposition of Collagen, but still keep their capacity to activate. These novel culture methods will now be used to test compounds for their pro-fibrotic capacity.

*ii. Transcriptome of fresh and cultured cells*

We determined changes in the transcriptome associated with human HSC activation in culture. We compared freshly isolated, uncultured qHSCs with culture-activated HSCs (aHSCs). Over 2,000 genes are differentially

expressed upon HSC activation (2,017/20,816 genes). This profiling allowed the confirmation of genes known to be associated with HSC activation, such as GREM1, LOX, TNC. This enabled identification of novel putative human *in vitro* HSC activation-associated genes. We find limited overlap (12-18%) between changes in gene expression underlying *in vitro* activation of mouse primary HSCs – the most common model used to study HSC biology – and human primary HSCs, suggesting a different transcriptional cascade underpinning HSC activation in both species. For example, neurotrimin (NTM), a quiescence associated gene in mouse HSCs, is upregulated during human HSC activation. We furthermore compared the transcriptome of activated and *in vitro* reverted human HSCs (by reversal medium) . Gene expression profiling experiments reveal that these *in vitro* reverted primary human iaHSCs display an intermediary phenotype that is distinct from qHSCs and aHSCs. Interestingly, this intermediary phenotype is characterized by the increased expression of several previously identified signature genes of *in vivo* inactivated mouse HSCs such as CXCL1, CXCL2, and CTSS, suggesting also a potential role for these genes in promoting a quiescent-like phenotype in human HSCs.

This study has been presented (oral/poster presentations) in different international liver meetings such as the 18th International Symposium on Cells of the Hepatic Sinusoid (Asilomar, California) and the 50th Annual Congress of The European Association for the Study of the Liver (Vienna, Austria). Moreover, this work has led to several publications in journals with high impact (Coll *et al.* Scientific reports 2015, El Taghdouini *et al.* Oncotarget 2015).

*iii. miRNAs in fresh and cultured cells*

Unveiling regulatory pathways of Hepatic Stellate Cells (HSC) quiescence and activation is essential to develop new therapeutic strategies to treat liver fibrosis. The aim of was to identify miRNAs regulating HSC activation by integrating HSC gene and miRNA profiling. miRNA analysis showed 215 up and 48 down-regulated microRNA in activated compared to qHSC. Transcriptome analysis identified 345 genes deregulated during HSC activation. Bioinformatic miRNA-mRNA integration revealed a set of deregulated miRNAs that presented a significant correlation with the expression of their predicted target genes. Expression of miRNAs regulating key genes involved in quiescence maintenance and in activation was confirmed in whole healthy and cirrhotic human liver. miRNA-inhibitors for miR-21 and miR-100, upregulated in aHSC, and mimic-miRNAs for miR-192, associated to qHSCs, were transfected in cultured HSC demonstrating their role in the regulation of key genes involved in HSC activation and fibrogenic phenotype. Integrated analysis of miRNA and gene expression profiles of qHSC and aHSC identified a miRNA signature of hepatic stellate cell activation and potentially liver fibrosis.

*iv. Epigenome of fresh and cultured hepatic stellate cells*

To evaluate whether activation of HSCs can be correlated with methylome changes we compared changes in the promoter methylome of aHSCs vs qHSCs. MeDIP-chip reveals 5,862 methylated genes in qHSCs and 5,191 in aHSCs. We identified a core overlap of ~50% of all methylated genes in either qHSCs or aHSCs. We further noted a net reduction in promoter methylation in aHSCs, with demethylation of 3,102 promoters (53% of all methylated promoters before culture), including several different members of the collagen and lysyl oxidase gene families, the main constituents and enzymatic stabilizers of fibrotic scar tissue. 47% of all methylated promoters in aHSCs was de nove methylated. We furthermore investigated the correlation between DNA methylation and gene expression and found 416 genes with concordant changes in DNA methylation and gene expression upon HSC activation. For these genes, transcriptional upregulation correlated with abrogation or reduction in promoter methylation, as shown for *ACTG2*, *LOXL1*, *LOXL2* and *COL4A1/2*. Conversely, transcriptional downregulation among these 416 genes was associated with DNA hypermethylation (*APOB*, *ADAMTS9*, *MMP15* and *CXCL9:* El Taghdouini *et al.* Oncotarget 2015).

**3.1.1.2. Liver sinusoidal endothelial cells**

To provide a possible source of endothelial cells (ECs) for the bioreactor, we established 3 methods to isolate ECs from human liver biopsies. Two methods were based on FACS sorting using combinations of positive (Tie2 or CD32B) and negative (CD45, podoplanin and/or UV) selection markers and 1 was based on macromolecule uptake, a distinguishing functional feature of LSECs. While the FACS-based methods were suitable for (comparative) transcriptomic profiling, LSECs could only be cultured when isolated by the function-based method. The transcriptome of sorted LSECs was compared to that of stellate cells and hepatocytes (El Taghdouini *et al*. Oncotarget. 2015), as well as to ECs from 2 other organs (Coppiello *et al.* Circulation, 2015) in order to define a unique LSEC reference gene signature. Like for hepatocytes, a major bottle-neck in obtaining

sufficient numbers of primary LSECs is their limited expansion capacity. To overcome this problem, functionally isolated LSECs were upcyted, which significantly prolonged their proliferation capacity (up to 40 population doublings). Quantitative analysis of bona-fide LSEC marker gene expression however revealed that their LSEC marker expression dramatically dropped, and that this loss could only partially be prevented by metabolic arrest. Also the use of epigenetic modifiers (*e.g.*, HDAC inhibitors) did not prevent dedifferentiation. As a means to restore the gene signature, a method based on chemical induction was tested, but this only partially reinduced the expression of some LSEC markers, including *STAB2* and *LYVE1*. Nevertheless, upon improving the function-based derivation protocol (*e.g.*, by shortening the procedure's duration), dedifferentiation was less dramatic and scavenger activity remained significantly higher than in the currently available human LSEC lines from commercial sources and these cells retained fenestrations, a morphological hallmark of LSECs (Oie *et al.*, in preparation).

### 3.1.2. Pluripotent stem cell derived cells

#### 3.1.2.1. Hepatocytes

During the tenure of HeMiBio the teams of C Verfaillie and Y Nahmias developed progressively improved methods to create hepatocytes from PSCs. As shown in **Figure 1**, this included optimizing the culture medium, the type of growth factors added, and mimic the vena porta blood composition early after birth. Despite these optimisations, the final cells generated from PSCs did not attain the maturity of primary adult liver derived hepatocytes, for which we term the cells hepatocyte like cells (HLCs). Nevertheless, we demonstrated that cells can be used for toxicity studies including molecules shortlisted by the SEURAT-1 consortium in the gold compound list, as well as list earmarked by the SEURAT-1 consortium as the ab initio drugs to be tested. In addition, we demonstrated that the HLCs can be infected with different hepatotropic viruses.

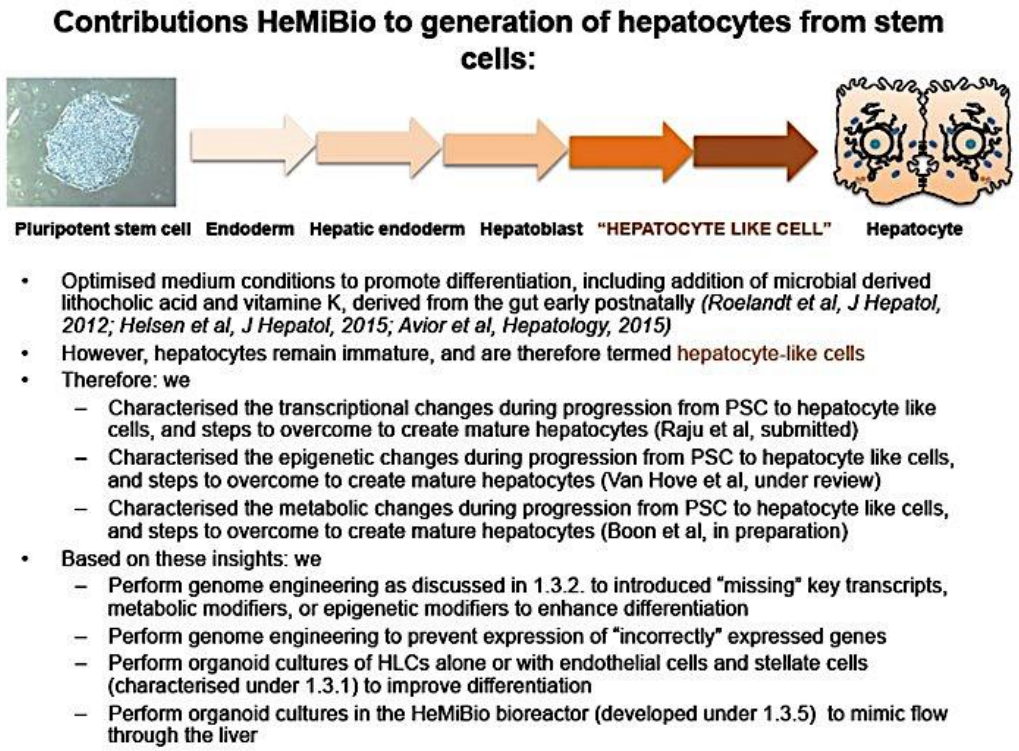



**Figure 1:** Hepatocyte differentiation from PSCs: Maturity attained is not fully similar to primary hepatocytes. Hurdles in the differentiation process have been identified which are now being addressed using different tools developed under HeMiBio

In view of the fact that the HLCs were not fully comparable with primary hepatocytes, extensive "omics" studies were done to characterize the progressively maturing hepatocytes and compare the final hepatocyte like cells with primary hepatocytes. Based on these insights ongoing studies are testing whether addressing the different hurdles present to create more mature functional hepatocytes can be addressed using for instance the genome engineering tools developed in HeMiBio (see 3.2).

In addition, with the creation of the HeMiBio bioreactor, as well as the characterization of liver sinusoidal endothelial cells and stellate cells (see 3.1), ongoing studies are evaluating if coculture of the HLCs with these non-parenchymal cells in 3D organoids, in the HeMiBio bioreactor (see 3.6), will allow full maturation of HLCs.

### 3.1.2.2. Hepatic stellate cells

We have developed for the first time a protocol to differentiate iPSC to HSC-like cells. iPSC progeny is enriched in PDGFRβ-positive cells and express typical HSC markers. The potential applications of iPSC-derived HSC-like cells to assess a fibrogenic response or toxicological assays are now being investigated.

*Differentiation of pluripotent stem cells to hepatic stellate cell-like cells:*
Pluripotent stem cells were directed to mesodermal cells, followed by the acquisition of a mesenchymal phenotype, mesothelial and finally the HSC-like phenotype. Flow-cytometry analysis showed that along differentiation, the percentage of PDGFR-beta cells (a good marker for HSCs) increased, reaching a maximum of ± 80% at day 12, suggesting that the majority of the differentiated cells have already acquired HSC phenotype.

*iPSc-derived HSC-like cells Characterization*
iPSC-differentiated HSC-like cells expressed ACTA2, Vimentin, PDGFRbeta, NGF and NCAM at a comparable level to primary HSC, as well as ALCAM, Desmin, glial fibrillary acidic protein (GFAP), PDGFRα, PDGFRβ, peroxisome proliferator-activated receptor (PPAR)γ, synaptophysin than primary HSC. Interestingly, markers associated with HSC activation such as ACTA2 and COL1α1, where expressed at a lower level in differentiated HSC-like cells, suggesting that their level of activation may be lower and could better resemble an intermediate activated phenotype with more quiescent cell features.

*Transcriptomic analysis of PSC-derived HSC-like cells:*
We performed transcriptomic analysis of primary fresh and activated HSCs and PSC-derived HSC-like cells at passage 1. As shown in **Figure 2**, principal component analysis, showed a clear clustering of each group of cells. Moreover, PSC-derived HSC-like cells showed an intermediate phenotype between quiescent and activated HSCs, although they clustered more closely to cultured activated HSC, suggesting that this expression profile could be due to culture conditions. Hierarchical cluster analysis also demonstrated as from the hepatocytes described in the section above, that PSC-derived HSC-like cells differ from primary quiescent HSCs (data not shown). These differences may be related to an incomplete differentiation or to the culture effect, since quiescent HSC where analyzed directly after isolation from human liver tissue.

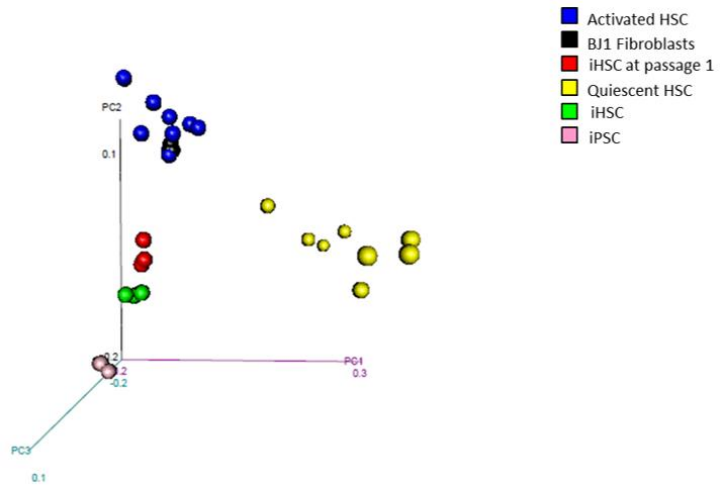



**Figure 2:** Transcriptomic analysis of iPSC-derived HSC. 3D Principal Component Analysis (PCA) showing distances between the transcriptomic profiles of different human cell types. Human samples are placed in a three-dimensional space according to their transcriptomic signature. Activated HSC are represented in blue (n=7), quiescent HSC in yellow (n=7), iPSC-derived HSC in green (n=3), cultured iPSC-derived HSC at passage one in red (n=3), iPSCs (n=3) in pink and parental fibroblast BJ1 in black (n=3).

### 3.1.3.3. Liver sinusoidal endothelial cells

We found that exposure of pluripotent stem cells (PSCs) to conditions favoring hepatocyte differentiation (Roelandt *et al., J Hepatol, 2012*) also generated cells with expression of EC markers, including Tie2 and CD31. Characterisation of the EC component isolated from hepatic PSC differentiation cultures after 3-4 weeks (based on the FACS sorting protocols designed for primary LSEC derivation described above), revealed that the yield of ECs was low (not more than 1% of total cells was $Tie2^{+}CD31^{+}$). After 45 days, no $Tie2^{+}$ cells remained. The expression profile of the $Tie2^{+}CD31^{+}$ cell fraction was immature, as most of the LSEC markers (*e.g.*, *LYVE1*, *STAB1*, *STAB2*, *CLEG4G*, *L-sectin*) were not or only marginally expressed. The resulting EC component was also heterogeneous, with some cells occasionally expressing CD32B protein, a scavenger receptor uniquely expressed in LSECs. Those cells sorted based on CD32B protein surface expression had a different gene signature from those sorted based on the Tie2/CD31 combination. Whether this differential expression pattern

represents the existence of LSEC zonation, a phenomenon well-documented for hepatocytes, remains to be determined. To improve the yield of ECs, therefore, an alternative differentiation method was designed wherein PSCs are committed to mesoderm and then to ECs. This resulted in a significant enrichment (up to 60%) of $KDR^+$ mesodermal progenitors, which then could be fated to $CD31^+$ ECs by exposure to EC growth factors, with a yield of up to 25% of total cells. Currently, the system is being optimised to limit contamination with fibroblasts that tend to overgrow the differentiated EC cultures. While this $CD31^+$ fraction already showed a significant induction in expression of bona fide LSEC markers (*e.g.*, *CD32B*, *L-SIGN* and *L-sectin*), currently a protocol based on forced transcription factor overexpression from the *AAVS1* locus (Ordovas et al., Stem Cell Reports 2015) is being tested to boost the specification process.

### 3.1.3. Upyte hepatocytes

Although upcyte® hepatocytes were already generated before the start of the project, in the course of the project the process to generate upcyte® hepatocytes has been improved and upcyte® hepatocytes have been generated from multiple donors. In addition upcyte® hepatocytes have been extensively characterized by Medicyte and HUJI with respect to functional activities (phase I, phase II, transporter activities), epithelial markers and application in toxicity testing (Levy *et al.* Nat. Biotechnol 2015). KUL-1A confirmed phase I activities of upcyte® hepatocytes.

Upcyte® hepatocytes from different donors including a donor from partner 11 were generated at Medicyte produced as working cell bank and delivered to HeMiBio partners. The cells were extensively characterized at partner P5 and P9 with respect to hepatocyte specific functions. Both partners could demonstrate that these cells exhibit similar metabolic functions to primary plated hepatocytes. Furthermore, upcyte® hepatocytes generated from different donors have been characterized further with respect to the response to hepatotoxic chemicals from the ToxBank list and non-hepatotoxic compounds. Upcyte® hepatocytes derived from different donors differed only slightly in their response to the compounds. All compounds could be correctly classified as non-toxic, moderate and toxic compounds which is in accordance with in vitro data from primary hepatocytes.

Comparison of whole genome expression profiles of primary hepatocytes and upcyte® hepatocytes revealed difference in the range of 1-2% of genes that were found to be more than twofold up- or downregulated. A significant portion of the affected genes are involved in cell growth which is one of the main differences in primary vs. upcyte® hepatocytes. Comparing upcyte® hepatocytes from the same donor cultured in two different labs revealed almost no statistical difference (0.25% of more than 2-fold differentially regulated genes).

### 3.1.4. Generation of liver sinusoidal endothelial cells from other cell populations

Since we anticipated that using primary LSECs or LSECs differentiated from PSCs may be subject to practical difficulties, in parallel we also worked on exploring alternative sources to generate the EC component of the liver bioreactor. We optimised a protocol to derive endothelial progenitors from the mononuclear fraction of peripheral blood and cord blood, known as blood outgrowth endothelial cells (BOECs). These cells have very low expression levels of LSEC-specific markers and do not exhibit uptake of macromolecules nor do they have fenestrae. Although BOECs have a significantly increased expansion potential compared to primary ECs, the number of passages they can undergo is too limited to make the cells amenable to genome engineering to build in molecular sensors, as we planned in PSCs. Therefore, BOECs were successfully upcyted such that passage frequency was increased up to 40 without alteration of EC marker expression. Before using these cells as a substrate for LSEC generation, approaches to induce an LSEC gene signature were first tested in human umbilical vein ECs (HUVECs). The chemical induction protocol was slightly less efficient in inducing LSEC marker expression in BOECs as compared to upcyted LSECs (see above). Currently, a second approach based on forced overexpression of a set of transcription factors identified during the comparative transcriptomic profiling in different EC types from different organs is being tested.

## 3.2. Genome engineering of iPSC

We created genome engineered pluripotent stem cells with a flippase recombination cassette in the AAVS1 locus to allow quick and easy (±3 weeks without need for single cell selection) inclusion of toxicity readout cassettes for non-invasive assessment of cosmetics and drug toxicity. (Ordovás L, *et al.,* Stem Cell Rep. 2015)

Tools for rapid and efficient transgenesis in "safe harbor" loci in an isogenic context remain important to exploit the possibilities of human pluripotent stem cells (hPSC). We created hPSC master cell lines suitable for FLPe recombinase-mediated cassette exchange (RMCE) in the *AAVS1* locus that allow generation of transgenic lines within 3-4 weeks with 100% efficiency and without random integrations. Using RMCE, we successfully incorporated several transgenes useful for lineage identification, cell toxicity studies, and gene over-expression. In **Figure 3** we summarize the concept and possibilities

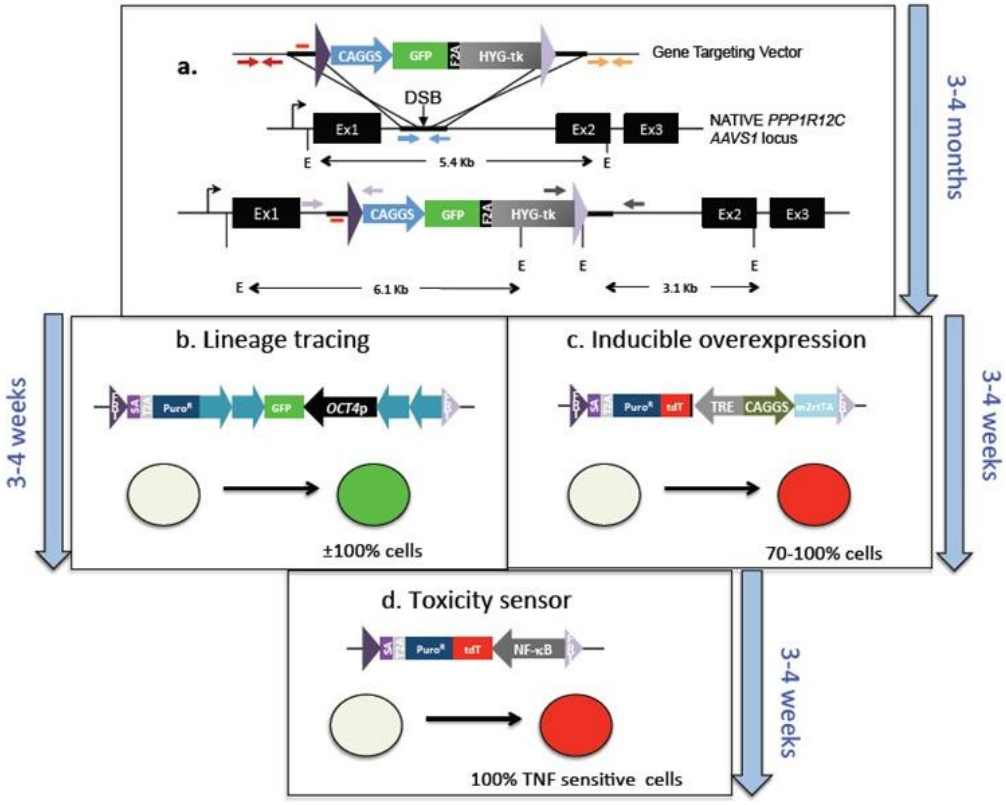



**Figure 3:** Creation of master cell line as well as lineage tracing, inducible over-expression and toxicity sensor line by incorporation of a Flippase cassette in the AAVS1 line: **(a)** Creation of the master cell line using zinc finger nucleases and a FRT flanked cassette including a positive selectable cassette as well as the thymidine kinase cassette, **(b)** Incorporation of an Oct4 promoter by flippase recombination allows identification of ±100% of pluripotent stem cells, **(c)** incorporation of an inducible tDt cassette allows for induction of 70-100% of the included gene sequence, **(d)** Incorporation of an NfkB sensor allows faithful detection of TNFα toxicity.

## 3.3. Electrophysical sensors

### 3.3.1. O2 sensor

The integrated optical oxygen sensor is based on fully biocompatible microbeads of polystyrene with a diameter of 50 µm (CPOx-50-RuP, Colibri Photonics, Germany). The beads are equipped with an oxygen-sensitive, phosphorescent ruthenium-phenanthroline-based dye. As oxygen acts as a quencher and leads to a decrease in decay time and signal intensity with increasing concentration (**Figure 4A**), the measurement of the decay time allows the determination of the ambient oxygen concentration. We chose to measure decay time rather than signal intensity, as the decay time is not sensitive to changes in probe concentration or excitation intensity over the course of the experiment, i.e. insensitive to loss of optical focus (**Figure 5**). To this end, the OPAL system (Colibri Photonics, Germany) was used for phase modulation, providing sinusoidal amplitude-modulated green

light (532 nm) for excitation (**Figure 4B**). Emission light is shifted in phase due to oxygen quenching (**Figure 4B**). In-phase background signals were eliminated using two-frequency phase modulation, i.e. excitation was performed with two frequencies in superposition.

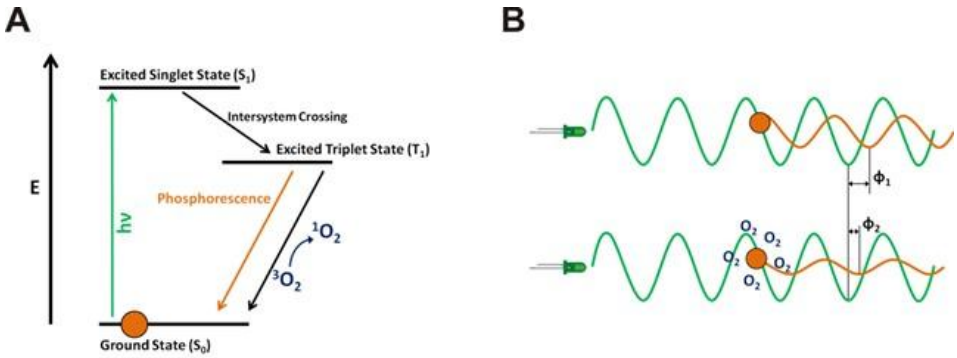



**Figure 4:** Principle of CPOx-50-RuP-based phosphorescence under modulated excitation. (A) Jablonski Diagram describing the energetic pathway of phosphorescence generation. The phosphorescence signal of the probe is emitted with a delay given by the lifetime of the excited triplet state $T_1$ of the dye molecule. Oxygen acts as a quencher as it is converted from ambient triplet to singlet oxygen trough energy uptake. This leads to a decrease of the decay time and signal intensity with rising oxygen concentration. (B) Scheme of the sinusoidally intensity-modulated excitation signal (green) used in the presented system to determine the decay time in real time. The emission signal (orange) exhibits an oxygen concentration-dependent phase shift with respect to the excitation signal. The shift can be correlated to the decay time and, thus, is used to determine the ambient oxygen concentration.

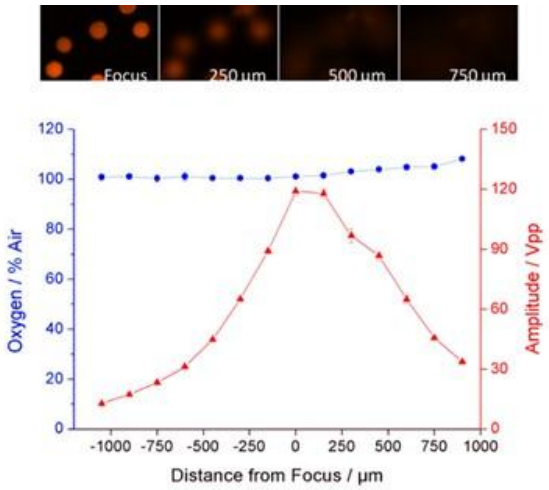



**Figure 5:** Phosphorescence decay time-based oxygen measurement is independent of signal intensity. Stepwise changing of the optical focus, leading to consecutively less signal intensity, has no influence on the measured oxygen concentration (nominal oxygen concentration atmospheric, *i.e.* constant).

### 3.3.2. ALT sensor

An amperometric sensor has been developed for the detection and the quantification of ALT. The sensor's technology is based on screen-printed electrodes. The equations below report the principle of detection of the ALT sensor: ALT generates glutamate in presence of the L-alanine and α-ketoglutarate. Thus when L-alanine and α-ketoglutarate are added in the proper concentrations, this enables a linear relation between the activity of ALT and the concertation of glutamate. The detection principle relies on the detection of glutamate through the reaction catalyzed by glutamate oxidase, thus making the ALT sensor an indirect sensor.

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \xrightarrow{ALT} \text{L-glutamate} + \text{pyruvate}$$

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{Glutamate\ oxidase} \text{⍰-ketoglutarate} + NH_3 + H_2O_2$$

ALT activity has been measured in HepG2 cell lysate as a function of cell concentration.

**Figure 6** shows the results of these measurements: using the ALT sensor developed within this project, it is possible to detect ALT from lysate for a concentration of cells of approx. $1.10^6$ cells/mL, with a corresponding activity of ALT in the order of 1.5U/L. It has to be noted that the measured ALT concentration is low compared

to most *in vivo* data found in literature. However data on ALT concentration in-vitro found in the literature vary from units per liter to thousands of units per liter, getting difficult to find a significant comparison.

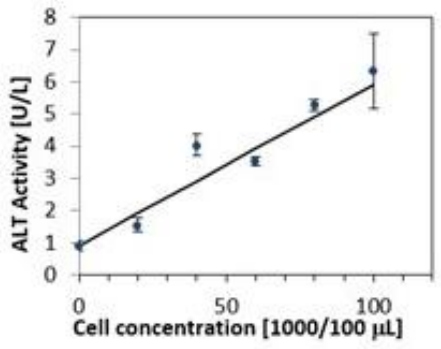



**Figure 6:** ALT measurements in HepG2 cell lysate. ALT activity is plotted as a function of cell concentration (in thousands per 100 µL) in the lysate. All samples were tested in triplicates.

## 3.3.3. Incorporation of sensors

### 3.3.3.1. Oxygen sensor

The oxygen-sensing microbeads were integrated directly into the reactor-internal cell culture compartment. This was realized by employing an optimized protocol for cell seeding leading to a three-dimensional, collagen-based cell culture matrix. By this, real-time oxygen detection in the direct micro environment of the cells was possible in a non-invasive fashion (**Figure 7A**). Commercially available electrochemical sensors for glucose and lactate detection in whole blood (BST GmbH, Germany) were adapted for the use in a cell culture environment by transferring them into a microfluidic flow cell (**Figure 7B**). The latter was addressed with cell culture medium using a fully automated fluidic system consisting of several solenoid valves and peristaltic pumps (**Figure 7C**). Thus, electrochemical signals were automatically recorded and stored for subsequent data processing steps.

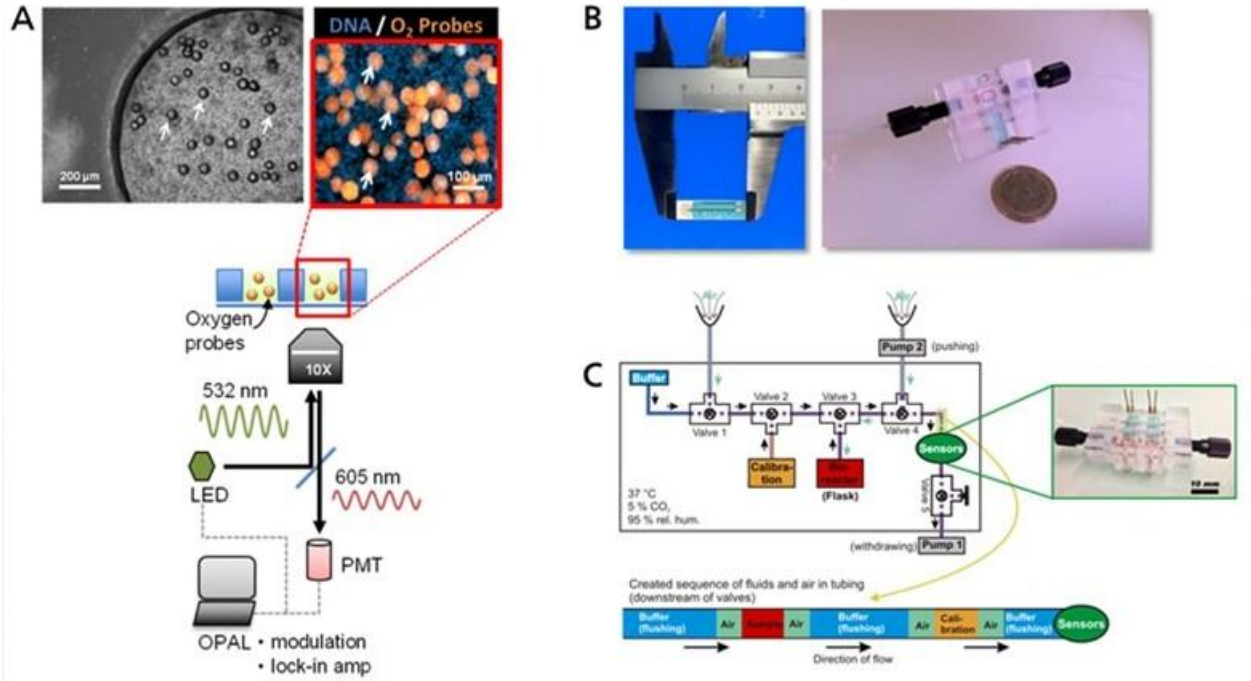



**Figure 7:** Integrated sensors. (A) Scheme of the measurement setup connected to the microscope, comprising an electronic control unit for signal modulation and readout, a LED for excitation, an optical filter set (531/40, 555, 607/70 nm), and a detector unit containing a photomultiplier (PMT). (B) Electrochemical, enzyme-based sensors for glucose and lactate. (C) Automated fluidic system for cell culture medium sample handling and measurement.

### 3.3.3.2. Switchboard

To overcome flow and shear fluctuation complicating sensor synchronization, we designed an equi-pressure combinatorial mixer composed of a microfluidic switchboard and a passive mixer. The microfluidic switchboard

consists of 11 inlets and a common outlet that are regulated by self-addressable micromechanical valves. The valves are controlled by one pressure manifold, delivering precise combinations of fluids to the mixer unit. Flow is driven by positive pressure provided by a second, independently controlled pressure manifold.

Using this setup we were able to automate sampling and calibration of a lactate electrochemical sensor and monitor hepatic toxicity, in real time:

Human Huh7 cells were seeded in a microfluidic chamber, producing a confluent monolayer. Cells were exposed to 100 µM of rotenone. Perfusate was connected to a high resistance waste syringe and a single inlet of the microfluidic switchboard for automated sampling (**Figure 8a**). We used the microfluidic switchboard to perfuse a sequence of buffer, air, sample and air over an enzymatic-amperometric lactate sensor every 2 h (**Figure 8b-c**). Introduction of air acts as a diffusion barrier preventing sample contamination and providing zero-point calibration. Between each experiment a calibration medium was introduced to correct sensor drift. The electrochemical sensor was connected to an embedded potentiostat. A fully automated 16.5 h experiment was programed allowing the derivation a time-of-death of 5.5 h (**Figure 8d**).

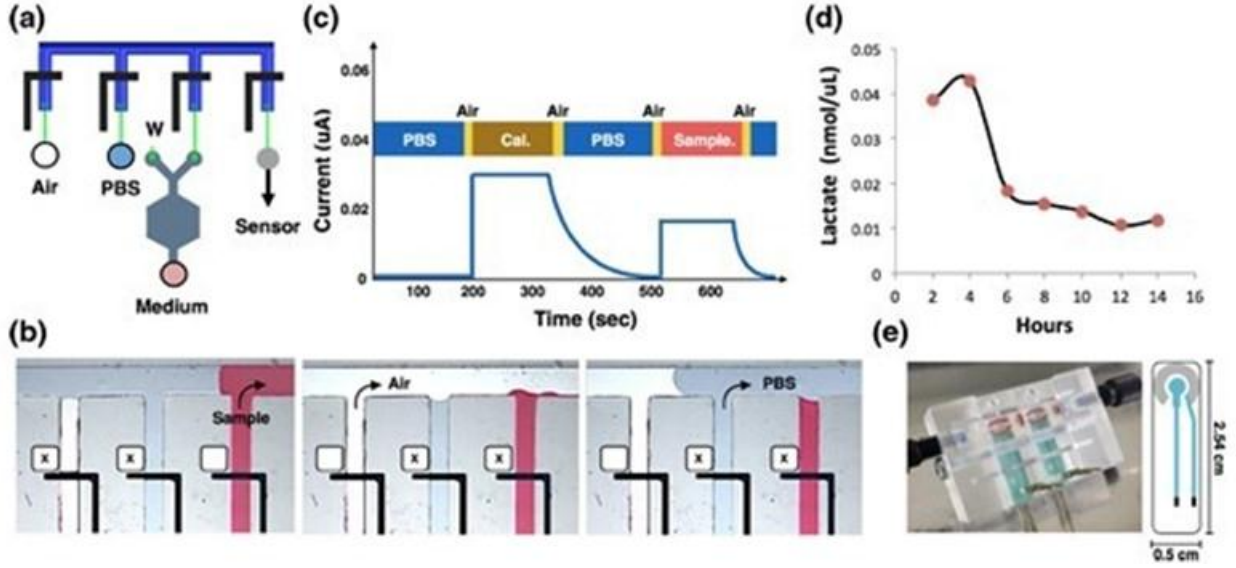



**Figure 8:** Real time monitoring of hepatic toxicity using automated sampling and calibration of electrochemical sensors a Flow system schematics: microfluidic switchboard (blue) connected to air (white), phosphate buffer saline (gray), **(a)** single outlet of the liver bioreactor, and the electrochemical lactate sensor (green). The second outlet of the liver bioreactor is connected to waste (W). Control valves are shown in black. **(b)** Sequential addition of sample (red), followed by air purging (white), and washing buffer (blue). Bar = 200 µm. **(c)** Readout from the sensor for a sequence of calibration fluid, buffer, air and a sample. The output current is proportional to the fluid Lactate concentration **(d)** Real time measurement of lactate production in perfusate of liver bioreactor exposed to 100 µM rotenone. Huh7 cells die within 5.5 h of exposure to the pesticide. **(e)** Image and schematics of the microfluidic lactate sensor and its low-volume PMMA housing

Thus, we demonstrate the generation of complex perfusion sequences, by connecting an electrochemical lactate sensor downstream of a liver bioreactor exposed to the environmental toxin rotenone. Our system permits automated sampling, washing, and calibration of the electrochemical sensor, providing continuous real-time measurement of cell viability.

### 3.3.3.3. Automated fluidic handling interface

A fluidic handling system capable of automatically pump, store and deliver the sample, washing and calibration solutions to the sensing module has been fabricated and tested. The interface has been designed to handle small volumes of sample and be compatible with the use of the bioreactors of HeMiBio (**Figure 9**).

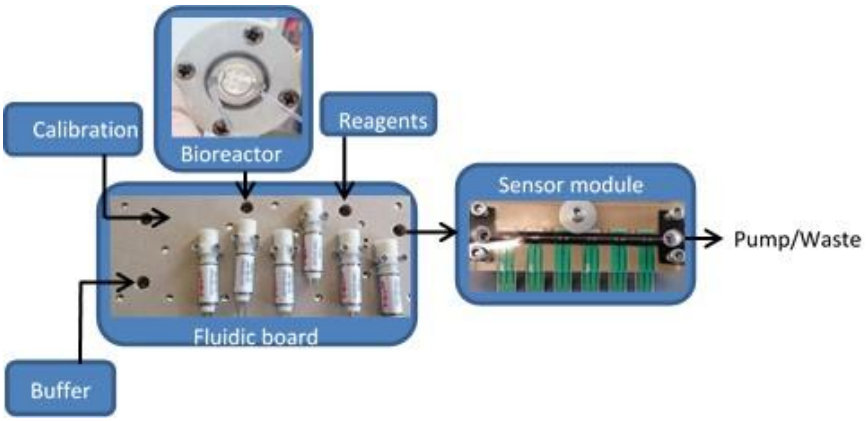



**Figure 9.** Schematic of the automated bioreactor monitoring setup.

The automated fluidic handling interface has been used to monitor glucose, lactate, ALT from the supernatant flowing out of the HeMiBio flow-over bioreactor, where a hepG2 cell culture has been exposed the cells to the toxic compound rotenone at a concentration of 200 M. Concentrations profiles of glucose, lactate, pO2, ALT were successfully recorded for five hours after the toxic insult with a sampling rate of 1 sample/hour. During these preliminary tests the cell necrosis was clearly visible by the lowering of oxygen consumption by the cells. At the same time, the ALT concentration signal showed a raise, indicating cell death (**Figure 10**).

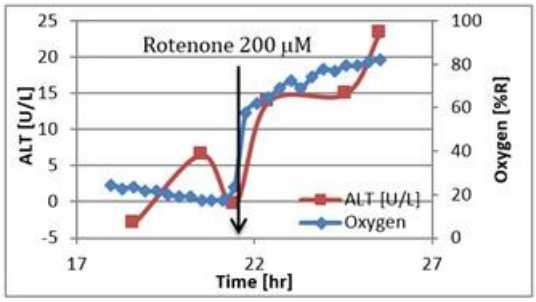



**Figure 10.** Concentration profile of pO2 and ALT in the HeMiBio bioreactor with a HepG2 cell culture before and after the exposition of the cells to 200 µM rotenone.

## 3.4. Patterning bioreactor

Cell-cell interactions play a key role in regeneration, differentiation and basic tissue function taking place under physiological shear forces. However, previous solutions to mimic such interactions by micro-patterning cells within microfluidic devices have low resolution, high fabrication complexity, and are limited to one or two cell types.

We designed and developed an antibody-based, microfluidic system capable of patterning any biotin-conjugated set of antibodies using streptavidin-based surface chemistry. The design permits the generation of arbitrary cell patterns from heterogeneous mixtures in microfluidic devices. This system is therefore capable of one-step cell isolation and patterning in microfluidic devices permitting the generation of tissue micro-patterns from complex cell mixtures with minimal sample preparation. Our approach can be broadly employed to combine cell isolation with micro-patterning. The significance of such a combination is the ability to directly inject a complex starting sample such as whole blood or tissue digest into a patterning device with minimal sample preparation, resulting in functional, perfusable tissue on a chip. Using this system we were able to demonstrate robust co-patterning of α-CD24, α-ASGPR-1 and α-Tie2 antibodies for rapid isolation and co-patterning of mixtures of hepatocytes and endothelial cells (**Figure 11**). In addition to one-step isolation and patterning, our device permits step-wise patterning of multiple cell types and empty spaces to create complex cellular geometries *in vitro*.

In conclusion, we developed a microfluidic device that permits the generation of perfusable tissue-like patterns in microfluidic devices by directly injecting complex cell mixtures such as whole blood or tissue digests with minimal sample preparation.

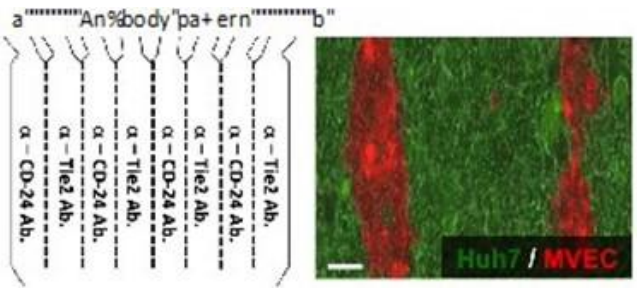



**Figure 11: (a)** Schematic diagram of antibody pattern used to capture and co-pattern hepatocytes and endothelial cells under flow. **(b)** Capture and co-patterning of Huh7 and MVEC on an anti-CD24/Tie2 patterned device at shear stress of 0.8 Pa. Under these conditions Huh7 show preferential binding. Scale bar = 100 µm.

## 3.5. Flow-over bioreactor

Microfluidic liver-on-chip devices offer an alternative to animal experiments as they can mimic the native microenvironment and support long-term function under continuous perfusion. One critical advantage of microfluidics is the ability to expose cells to a stable stimulation over time, eliminating the rapid loss of signal due to nonspecific adsorption and metabolism that characterizes both static *in vitro* assays and *in vivo* experiments

*Bioreactor design and characterization*

The liver is highly vascularized, delivering oxygen at rates of 0.9 nmol/s/$10^6$ cells, while at the same time protecting hepatocytes from the negative effects of shear forces. The gradient of oxygen that develops along the sinusoid is thought to induce a demarcation of function, termed *metabolic zonation.* To mimic this environment, we designed a stainless steel bioreactor that protects hepatocytes from shear forces while creating stable oxygen and nutrient gradients mimicking the *in vivo* zonated microenvironment (**Figure 12a-g**). HepG2/C3A cells were suspended in collagen gel prior to seeding directly in the microwell insert (**Figure 12c**). The small diameter of the microwells leads to rapid aggregation, creating liver spheroids after overnight incubation. The inserts were physically sealed in the bioreactor and continuously perfused thereafter. Computation fluid dynamic modeling (**Figure 12d–g**) showed physiological shear forces under 0.1 Pa inside the microwells for perfusion rates of 10 µl/min. The high flow rate resulted in similar oxygen concentration delivered to each well in the array (**Figure 12e, f**). Oxygen consumption caused a gradient to develop along the perfused spheroid mimicking the *in vivo* microenvironment.

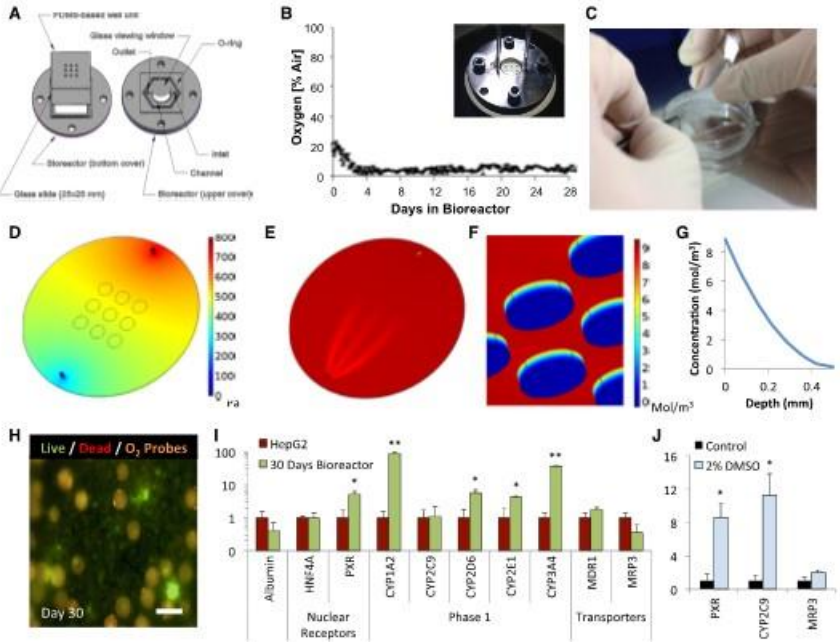



**Figure 12 (a)** Bioreactor components in overview. *Left* bottom part with PDMS microwell insert on glass substrate; *top view. Right* top part with glass coverslip for optical accessibility also from top, recess for O-ring and perfusion inlet/outlet; *upside down view.* **(b)** Long-term oxygen measurement over 1 month in bioreactor perfused with fresh cell culture medium at 10 µl/min. 100 % air represents atmospheric dissolved oxygen concentration (21 % O2 = 210 µmol/l; no consumption) **(c)** photograph taken during microwell insert seeding procedure. **(d)** numerical simulation of the shear stress magnitude and **(e, f)** oxygen concentration along the bioreactor. **(g)** Oxygen concentration variations within the well (from *top* to *bottom),* mimicking the *in vivo* microenvironment. **(h)** Fluorescent staining for metabolically active cells (green) and dead nuclei (red) after 28 days of continuous perfusion in the bioreactor. Ru-CPOx beads emit orange. *Bar* 100 µm. **(i)** Log-scale gene expression analysis of HepG2/C3A cells in static culture compared with those perfused for 28 days. PXR and CYP3A4 expression shows a fivefold and 36-fold increase in the bioreactor, respectively. **(j)** Cells exposed to 2 % DMSO for 28 days show marked elevation of PXR and its target genes CYP2C9 and MRP3. *Error bars* indicate standard deviation for all panels. *p < 0.05, **p < 0.01 by student *t* test.

*Long-term maintenance of perfused spheroids*

HepG2/C3A cells were maintained for over 28 days *in vitro*, while displaying over 98% viability (**Figure 12h**) and high expression of liver specific markers including CYP450 enzymes as compared to static culture (**Figure 12i**). Remarkably, CYP3A4 showed a 36-fold higher expression than in static culture, reaching 6% of primary human hepatocyte level (**Figure 12i**) Finally, continuous exposure of the HepG2/C3A spheroids to 2 % DMSO for 28 days resulted in a further eightfold increase in PXR expression, and an associated twofold and tenfold increase in its target genes CYP2C9 and MRP3, respectively (**Figure 12j**).

## 3.6. 3D bioreactor

In the framework of HeMiBio, a 3D microfluidic bioreactor for hepatic cell cultures was developed. The bioreactor is constructed using Cyclic Olefin Copolymer (COC), since the material has good chemical resistance, low adsorption and good optical properties, including low auto-fluorescence. To guarantee these desirable properties for the finished bioreactor, after structuring the microchannels, the COC is bonded without adhesives. Two parallel approaches were developed for structuring the COC. In a first approach, mechanical micro-milling of the channels allows for extremely fast manufacturing of new design variations, at the expense of difficulties in scalability to mass-production and a channel surface that requires post-processing to achieve sufficient optical quality. In a second approach, hot embossing using epoxy molds allows for direct structuring of optical grade channels and is scalable to mass production, at the expense of longer cycle time in the development of new channel designs. In both cases, the structured COC is bonded using thermocompression to seal the channels. The design of the bioreactor was intended to maintain and expose pre-formed hepatic co-culture spheroids to toxicants for more than a week. Once seeded, spheroids rest on a polycarbonate porous membrane, allowing the medium to flow-through, while flow-over is maintained to avoid an excess pressure on the cells. In a single bioreactor, 9 wells are connected to a common inlet to provide the cells with fresh culture medium or test compounds. Due to the challenging nature of lab-scale structuring and bonding of COC, the testing of the 3D bioreactor experienced more delay than expected, and testing with hepatic cells was limited to some small scale initial trials. Significant progress has been made towards producing a 3D COC-based bioreactor for hepatic cell culture, and most technological hurdles in producing prototype reactors have been overcome. Further testing is needed to see which improvements to the reactor design or the flow conditions should be made to ensure cell viability.

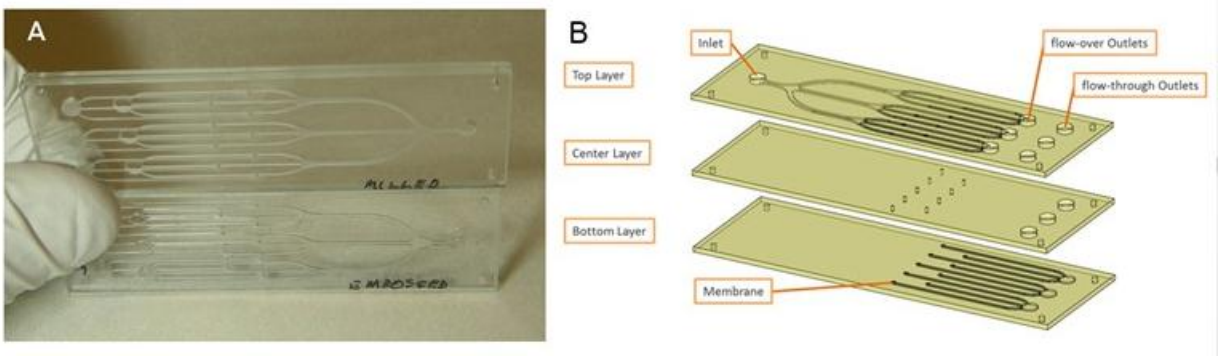



**Figure Figure 13: A** Camera picture of assembled milled (top) and embossed (bottom) 3D bioreactor. **B** Exploded view of 3D bioreactor design

## 3.7. Toxicology

### 3.7.1. AOP generation

The adverse outcome pathway (AOP) has played in a key role in SEURAT, and hence it was fully adopted in HeMiBio. An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level relevant to risk assessment. Since cholestasis, or bile acid accumulation, is a major form of liver toxicity that can be potentially induced by cosmetic ingredients, VUB-B focused their attention on the establishment of an AOP framework for cholestasis triggered by chemical-mediated inhibition of the bile salt export pump transporter protein. For this purpose, an

in-depth survey of relevant scientific literature was carried out in order to identify intermediate steps and key events. The latter include bile accumulation, the induction of oxidative stress and inflammation, and the activation of specific nuclear receptors. Collectively, these mechanisms drive both a deteriorative cellular response, which underlies directly caused cholestatic injury, and an adaptive cellular response, which is aimed at counteracting cholestatic insults. AOP development was performed according to Organisation for Economic Co-operation and Development (OECD) guidance, including critical consideration of the Bradford-Hill criteria for weight-of-evidence assessment and the OECD key questions for evaluating AOP confidence. The postulated AOP is expected to serve as the basis for the development of new *in vitro* tests and the characterization of novel biomarkers of chemical-induced cholestasis.

To trigger efficient exploitation of the bioassays by other HeMiBio partners, we developed standard operation procedures and organised a Winterschool entitled “Concepts of toxicology and risk assessment” in Barcelona-Spain on 16 January 2013 and a workshop entitled “Practical concepts of *in vitro* and *in silico* toxicology” in Brussels-Belgium on 4-6 June 2013 in order to teach theoretical aspects and provide a hands-on experience with aforementioned *in vitro* techniques.

In addition, some HeMiBio partners were also involved in other SEURAT projects, in particular DETECTIVE, which facilitated inter-project communication, especially with respect to selection of chemicals and toxicity testing in liver-based *in vitro* models. In addition, VUB-B acted as co-leader of the mode-of-action working group, overarching the SEURAT projects, and fed back on this on a routine basis to the HeMiBio consortium. Thus, HeMiBio not only significantly contributed to external dissemination of research results produced in SEURAT, but was also instrumental to maintain inter-project communication.

### 3.7.2. Gold compound list

*Selection Gold compound list*

At the start of SEURAT, VUB-B has selected a number of prototypical liver-inducing compounds based on a set of well-rationalized criteria, including mode-of-action, species specificity, bio-activation requirements, physico-chemical profile, overall compound documentation, and legal and safety aspects. As such, 12 compounds were selected that induce cholestasis (cyclosporine A, chlorpromazine hydrochloride, troglitazone), steatosis (valproic acid, acetylsalicylic acid, amiodarone) or apoptosis (diclofenac, acetaminophen, aflatoxin B1), or that served as negative controls (mannitol, menthol, melatonin). In order to select cosmetic ingredients to be tested in the second phase of SEURAT, and thus of HeMiBio, an extensive screening was performed of the safety evaluation reports of cosmetic ingredients of the Scientific Committee on Consumer Safety (SCCS). The VUB-B team has established an in-house databank of all SCCS reports published between 2000 and 2015. In essence, it was found that the liver is the most prominent organ potentially affected in repeated dose toxicity studies with cosmetic ingredients as described in the SCCS safety reports. The relevance of the liver as being the most frequently affected organ by the cosmetic ingredients was further scrutinized by listing all changes observed in histopathological and biochemical parameters that could point to hepatotoxicity. This was done in collaboration with several clinicians. Based on these combined observations, 7 cosmetic ingredients were identified as plausible liver steatosis-triggering candidates (3-methylamino-4-nitrophenoxyethanol, Basic Brown 17, HC Blue number 17, triclosan) or cholestasis-inducing candidates (2,7-naphtalenediol, Basic Red 51, triclosan) to be potentially tested in HeMiBio. In a later phase of the project, SEURAT requested the HeMiBio consortium to specifically focus on cosmetic ingredients that could cause liver fibrosis. Accordingly, a similar intensive exercise as outlined above was performed, yielding 4 potentially liver fibrosis-inducing cosmetic ingredients, namely furfural, benzophenone, Solvent Red 23 and cupric acetate/copper sulphate. Further on, SEURAT adopted another strategy and, in the context of their *ab initio* case study, requested all liver-oriented projects to focus on the testing of 3 compounds, namely valproic acid, methotrexate and piperonyl butoxide. These chemicals have therefore been prioritized for toxicity testing in HeMiBio.

#### 3.7.2.1. UpCyte hepatocytes

Upcyte hepatocytes were differentiated by removal of OSM from the culture medium, resulting in functionally polarized cells with albumin production, phase I and II gene expression, and CYP450 activity similar to those of

primary human hepatocytes. Upcyte hepatocytes retain the ability to modulate CYP450 activity, and expression through nuclear receptor activation, with different isolates exhibiting variable responses.

*Use of Upcyte hepatocytes for toxicological evaluation*

To assess the ability to predict toxicological outcomes, we determined the concentration that causes 50% cell death (TC50) over a 24h exposure to known hepatotoxic compounds (**Figure 14a**). A permutation test revealed that the TC50 toxicity profile of upcyte hepatocytes was not significantly different from that of primary hepatocytes. IVIVC of 23 additional compounds revealed an excellent correlation between all donors and primary human hepatocytes ($R^2$ = 0.99).

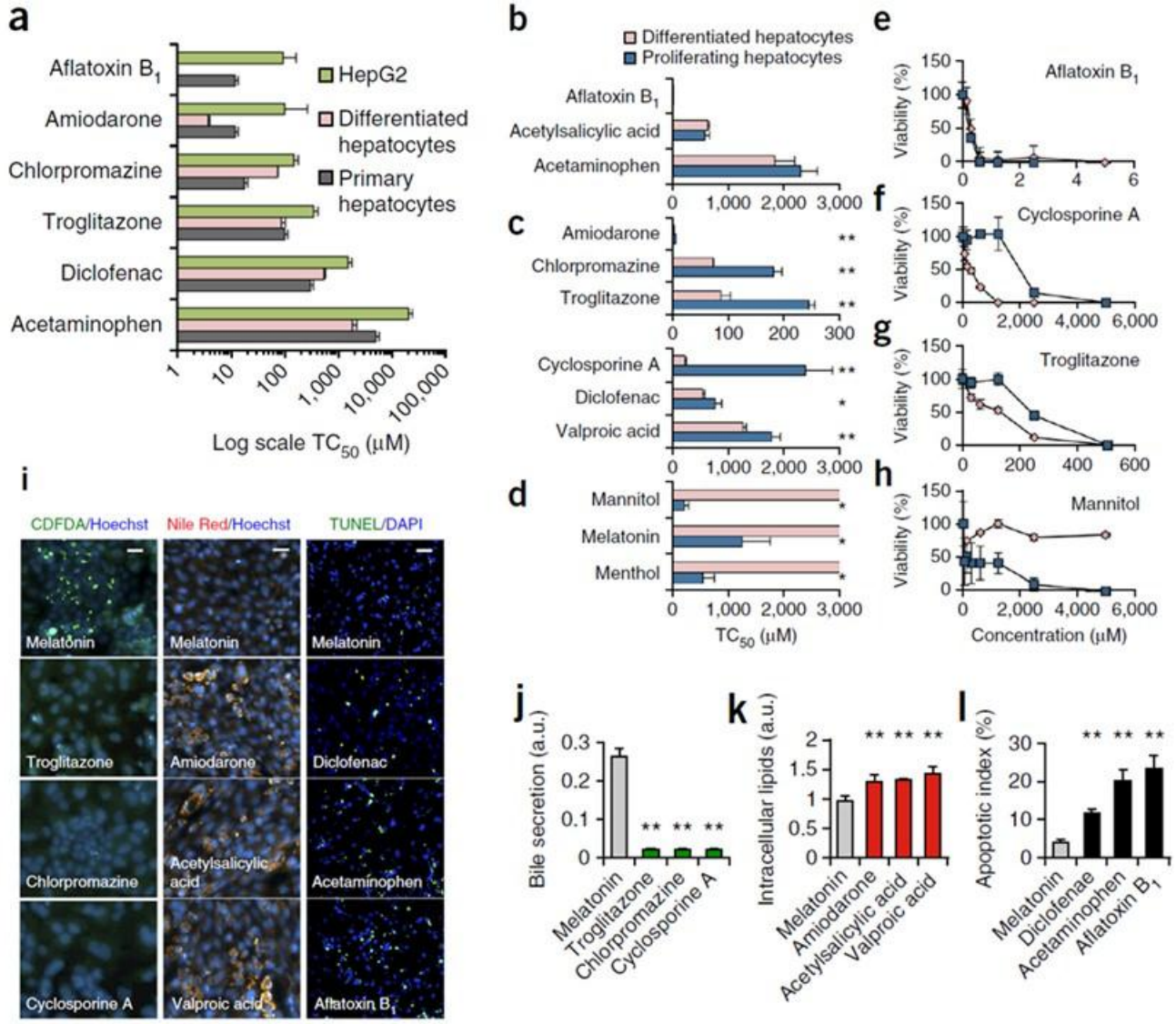



**Figure 14**: (**a**) TC50 values of six chemical compounds obtained from 24-h dose-response studies in differentiated hepatocytes, HepG2 cells and cryopreserved primary human hepatocytes. The normalized TC50 toxicity profile of differentiated hepatocytes was not significantly different from that of primary human hepatocytes ($P$ = 0.466; $n$ = 4), whereas the HepG2 profile was significantly different ($P$ = 0.030; $n$ = 3). (**b**–**d**) TC50 values of metabolically functional differentiated hepatocytes and proliferating hepatocytes of the same genotype and passage number ($n$ = 4). *$P$ < 0.05, **$P$ < 0.01 (**c**,**d**). (**e**–**h**) Dose-dependent toxicity curves of differentiated hepatocytes treated with various compounds, compared with proliferating hepatocytes of the same genotype and passage number. (**i**–**l**) Fluorescence quantification of adverse outcome pathway in differentiated hepatocytes. (**i**) Loss of bile acid production (cholestasis) evaluated by CDFDA staining, lipid accumulation (steatosis) by Nile Red staining and apoptosis by TUNEL labeling of nuclei. (**j**) Cholestasis in differentiated hepatocytes exposed for 24 h to cholestasis-causing drugs or melatonin (negative control) (**$P$ < 0.01; $n$ = 3). (**k**) Steatosis in differentiated hepatocytes after 48 h of exposure to steatosis-causing drugs or melatonin (**$P$ < 0.01; $n$ = 3). (**l**) Apoptosis of differentiated hepatocytes following 24 h of exposure to apoptosis-causing drugs or melatonin (**$P$ < 0.01; $n$ = 4). Data are from donor 653. a.u., arbitrary units; all $n$ values represent the number of biological samples analyzed per condition. All error bars indicate ± s.d.

Second, we compared the toxicological profile of proliferating and differentiated hepatocytes of the same passage (**Figure 14b-h**). The TC50 of compounds clustered into three groups. One group showed no difference between proliferating and differentiated cells (**Figure 14b**). The second group showed significantly higher toxicity in differentiated hepatocytes than in proliferating hepatocytes, suggesting metabolic activation was required for toxicity (**Figure 14c**). Surprisingly, the three control compounds clustered into a group in which toxicity was higher in proliferating than in differentiated hepatocytes (**Figure 14d**). Proliferating hepatocytes were affected by concentrations as low as 200 µM for mannitol (**Figure 14e-h**).

To test for adverse outcome pathways, we utilized TC20 concentrations of each drug (**Figure 14i**). We quantified bile secretion as a measure of cholestasis (**Figure 14j**). All three cholestasis drugs caused morphological changes leading to loss of bile secretion. Exposure to the steatosis-causing agents caused a 33–47% increase in intracellular lipids compared to control (**Figure 14k**). Finally, exposure of the cells to apoptotic drugs caused 3- to 6-fold increases in apoptosis compared to the control (**Figure 14l**). These data confirm Upcyte hepatocyte ability to predict toxicological endpoints providing a useful tool for predictive toxicology.

#### 3.7.2.2. PSC hepatocytes

Human pluripotent stem cells (hPSCs) represent an excellent source of differentiated hepatocytes; however, previously developed protocols still produce fetal-like hepatocytes with limited mature function. Striving to increase maturity of hPSC derived hepatocytes, and characterize their potential to fulfill different experimental needs, we took several avenues.

*Predictive toxicology*

Interestingly, fetal hepatocytes acquire mature CYP450 expression only postpartum, suggesting that nutritional cues may drive hepatic maturation. We established a differentiation protocol, which mimics postnatal nutritional cues. The Nahmias team was able to show that vitamin $K_2$ and lithocholic acid, a by-product of intestinal flora, activate pregnane X receptor (PXR) and subsequent CYP3A4 and CYP2C9 expression in hPSC-derived and isolated fetal hepatocytes. Differentiated cells produce albumin and apolipoprotein B100 at levels equivalent to primary human hepatocytes, while demonstrating an 8-fold induction of CYP450 activity in response to aryl hydrocarbon receptor (AhR) agonist omeprazole and a 10-fold induction in response to PXR agonist rifampicin. Flow cytometry showed that over 83% of cells were albumin and HNF4α positive, permitting high-content screening in a 96-well plate format. Analysis of 12 compounds showed an $R^2$ correlation of 0.94 between TC50 values obtained in stem cell– derived hepatocytes and primary cells, compared to 0.62 for HepG2 cells. Finally, stem cell–derived hepatocytes demonstrate all toxicological endpoints examined, including steatosis, apoptosis, and cholestasis, when exposed to nine known hepatotoxins. This body of work provides fresh insights into liver development, suggesting that microbial-derived cues may drive the maturation of CYP450 enzymes postpartum. Addition of these cues results in the first functional, inducible, hPSC-derived hepatocyte for predictive toxicology.

### 3.7.3. Liver fibrosis model

One of the HeMiBio achievements is the development of an *in vitro* drug-induced liver fibrosis model. As mentioned earlier, traditional HSC 2D mono-cultures lead to the activation of the HSCs towards fibrotic myofibroblast and does not allow testing of compounds that need metabolisation by hepatocytes or hepatotoxicity to cause liver fibrosis. The developed model consists of human 3D hepatic organoids comprised of HepaRGs® and primary human HSCs. The organoids are generated by mixing the two single-cell suspensions in a ratio of 1Heps:2HSCs in non-attachment round-bottom 96 well-plate wells with orbital stirring. This allows for quick generation of spheroids with an optimal diameter of ≤200 µm, representing the maximum physiological distance between a cell and a blood vessel and precludes the formation of necrotic cores. **Figure 15** shows a schematic representation of such spheroids, in which after several days in culture HSCs arrange themselves in the core of the spheroid while HepaRGs concentrate more at the outside (**Figure 15**, lower panel).

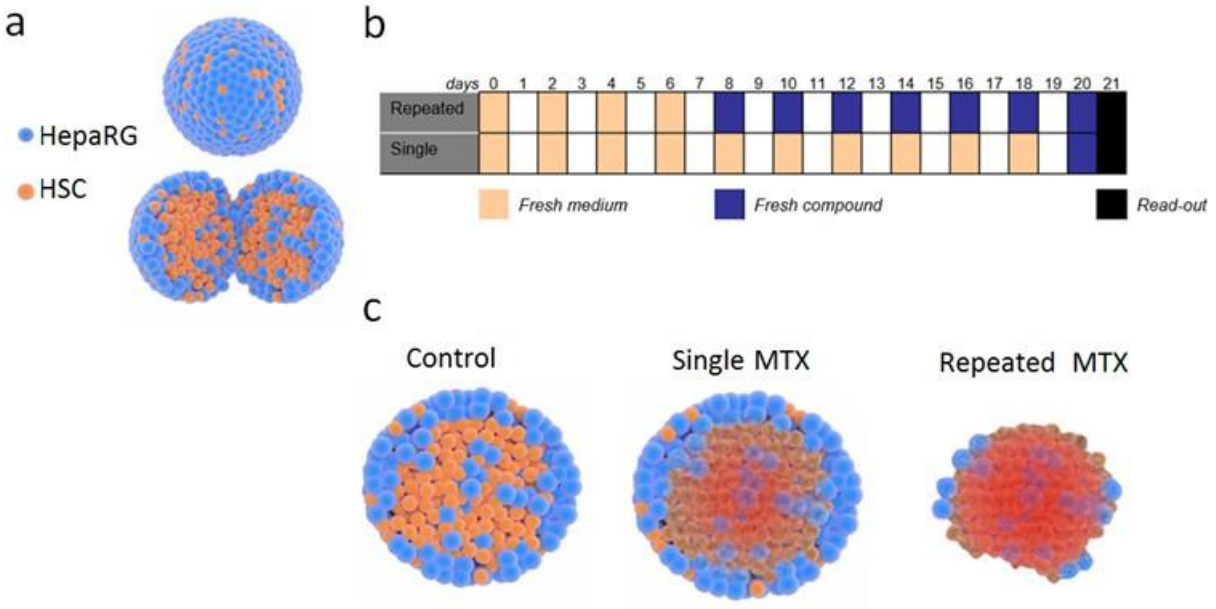



**Figure 15:** Schematic representation of the 3D hepatic organoids organization and read-out after exposure to pro-fibrotic compounds. **(a)** cellular distribution of HepaRG and Hepatic Stellate Cells (HSCs) within the organoid); **(b)** exposure scenario to compounds such as Methotrexate (MTX) and Allyl Alcohol during organoid culture time; **(c)** Schematic representation of HSC activation and collagen accumulation within the spheroid upon single and repeated exposure to MTX. Brown cells represent activated HSCs and the red color represents the accumulation of cross-linked collagen around the cells. Note that after repeated MTX treatment many hepatocytes have died and only the core remains which is mainly composed of activated HSCs that secrete and deposit high amounts of collagen. A single MTX treatment does not lead to hepatocyte death but also results in activation of HSCs in the centre of the spheroid (Leite *et al*. Biomaterials 2016).

In these hepatic organoids both hepatocytes and HSCs maintain their cell-specific functions/characteristics for at least 3 weeks. This is the first *in vitro* culture system that can detect drug-induced hepatotoxicity as well as drug-induced liver fibrosis. Its long stability allows not only single but also chronic repeated-exposure to reference fibrosis compounds such as Methotrexate and Allyl Alcohol. Organoids exposed to such compounds show an increase of collagen secretion and accumulation in the organoid, remeniscent of what happens in *in vivo* fibrotic livers. Even the hepatotoxic-independent HSC activation observed after repeated exposure to Allyl alcohol is hepatocyte-dependent since the effect is not observed in the HSC 3D mono-cultures (Leite *et al.* Biomaterials 2016). This highlights the potential of the hepatic organoids to not only "just" identify hepatotoxic compounds that might cause indirect HSC activation (i.e. MTX), but also by compounds that are not hepatotoxic but need the presence of functional hepatocytes to indirectly activate HSCs (i.e. Allyl alcohol at the tested concentrations). The relevance of this *in vitro* liver fibrosis model, developed entirely during the HeMiBio project, is that it allows the screening of pro-fibrotic drugs with human relevance before being tested in animals. Furthermore, this model can also contribute to the development of anti-fibrotic therapies, but might require further incorporation of liver cell types such as liver sinusoidal endothelial cells, Kupffer cells and Natural Killer cells.

### 3.7.4. Read across studies

In the last year of Seurat-1, HeMiBio actively participated in the read across study coordinated by Terry Schultz and Mark Cronin entitled "Read-across of 90-Day Oral Repeated-Dose Oral Toxicity for β-Olefinic Alcohols: A Case Study of Compounds with Similar Metabolism". The hepatic organoid cultures (HepaRG/HSCs) optimized during HeMiBio were assessed for fibrosis using a selection of β-unsaturated alcohols. Based on the results with 2-propen-1-ol, the 3D HepaRG/HSC co-culture model was used to evaluate five other □-unsaturated alcohols. The data obtained using single and repeated exposures (14 days) of the □-unsaturated alcohols reveal that while straight chain β-olefinic alcohols induce HSC activation (upregulation of mRNA levels of COL1A1, COL3A1 and LOXL), the vinylic methyl-substituted β-olefinic alcohols only weakly induce these markers. This *in vitro* data support the read across *ex vivo* and in chemico data in concluding that 2-propen-1-ol can be read-across to

other primary and secondary β-alkenols but a subcategorization is necessary. This work will be discussed in the ECHA Workshop April 18-20 in Helsinki (2016).

#### 3.7.5. Ab Initio studies

In the final year of Seurat-1, HeMiBio actively participated in the Ab Initio case study by testing Pyperonyl butoxide (PBO), the compound that was puth forth by Cosmetics Europe to be used in this level 3 case study. The compound was tested at different concentration in iPSC-derived hepatocyte like cells, in Upcyted-hepatocyte-like cells, in HepG2 as well as in hepatic 3D co-cultures of HepaRG and HSCs. Depending on the culture used, different readouts such as toxicity, function, mRNA levels of (hepatocyte) functionality or (HSC) activation markers was tested. The obtained data was communicated to Andrew White (Unilever) and Gladys Ouedraogo (L'Oréal) who coordinate this Ab Initio case study and part of the data was presented by them at the final Seurat-1 Symposium.

# 4. Potential impact and the main dissemination activities and exploitation of results

## 4.1. PSC derived cells

### 4.1.1. Hepatocytes

Human pluripotent stem cells (hPSCs) represent an excellent source of differentiated hepatocytes. We have explored and developed the potential of PSC derived hepatocytes in several avenues:

Predictive toxicology: The newly established hPSC differentiation protocol in the HUJI Labs towards hepatocytes produces hepatocytes like cells that are capable of demonstrating all toxicological endpoints examined, including steatosis, apoptosis, and cholestasis, when exposed to nine known hepatotoxins. Mimic $TC_{50}$ values of drugs with an $R^2$ correlation of 0.94 as compared to primary cells, and recapitulate the Hepatocyte ability to modulate CYP450 activity in response to transcriptional regulator agonists. This body of work provides means of producing *functional, inducible, hPSC-derived hepatocyte for predictive toxicology*.

This work has been published in "Hepatology" (Avior *et al.,* Hepatology, 2015). This work has been presented (oral/poster presentations) in over 10 international and local scientific meeting. It is a winner of the Presidential Poster Distinction at the AASLD conference, 2015.

Nevertheless, as has also been described by many other teams, although progress is being made in creating more mature hepatocytes with increased CYP levels among others, the cells still do not attain all functional, metabolic and transcriptional characteristics of primary mature hepatocytes. Future studies will still be needed to further optimize the differentiation to create fully mature hepatocytes, even if the studies during HeMiBio have made significant strides towards this goal.

### 4.1.2. Hepatic stellate cells

One of the main goals of HeMiBio is to engineer different liver cell type generated from induced pluripotent stem cells (iPSC) to be used in a liver-simulating device (Hepatic Microfluidic Bioreactor) mimicking the complex structure and function of the human liver. We have developed for the first time a protocol for generating hepatic stellate cell (HSC) derived from human iPSC. The iPSc derived cells have been functionally characterized. Generated HSC-like cells accumulate vitamin A in lipid droplets, up-regulate activation markers in response to pro-fibrogenic and pro-inflammatory stimulus and as the primary HSC, differentiated cells have the ability to migrate when they are activated. The potential applications of iPSC-derived HSC-like cells to assess a fibrogenic response or toxicological assays are now being investigated

This work will be presented in the 51st Annual Congress of The European Association for the Study of the Liver, Barcelona. Moreover, the invention of the protocol that describes the differentiation of induced pluripotent

stem cells towards hepatic stellate-like cells enriched population has been applied to be patented by KU Leuven and IDIBAPS. In addition, this work will be send for publication: Directed differentiation of IPS cells to hepatic stellate cells (Coll, Perea, Boon, Verfaillie, Sancho-Bru; manuscript in preparation).

## 4.2 Bioreactor

The developed "flow-over" or 2D bioreactor system for physiologically relevant cell culture and advanced metabolic real-time monitoring, by means of the integrated sensing technologies was shown to be of high relevance for the field of microfluidic liver equivalent devices and mechanistic, *in vitro* toxicology. The platform and the generated data were presented in numerous international congresses and symposia and published in various well-recognized scientific journals. Furthermore, US and EP-patent applications titled: "Method and System for Continuous Monitoring of Toxicity" were filed for this invention. The developed system provides the means to culture cells in microbioreactors for extended periods of time during which they can be exposed to various compounds in either single or repeated doses, all the while being monitored, in real time, for their oxidative and glycolytic metabolism, in order to assess their response to having been challenged by toxins. The mechanistic studies enabled by this system exceed the possibilities of *in vivo* experimentation, as the effects can be observed as they develop, i.e. kinetic data on drug-induced liver injury can be obtained. In contrast to experimentation in animals and conventional *in vitro* assays, that both represent the analysis of endpoints, the pathogenesis can be monitored and studied in real-time. This offers detailed mechanistic insights into the intracellular molecular events ultimately leading to the endpoint of liver injury. As an added benefit, the developed bioreactor gives the opportunity to design studies in a much faster and more cost-efficient way as compared to conventional assays. The intended exploitation comprises the sale of devices, the provision of services for in-house measurement series in the partners' labs and licensing.

With respect to the developed "flow-through" or 3D bioreactor, the development was still ongoing at the end of the project. The establishment of robust fabrication routines and reliability tests for leakage-free use required more time than initially anticipated. This impeded the regular use for larger scale cellular experiments and the generation of toxicological data using the 3D bioreactor. However, these steps would be the next to take at the current time point.

## 4.3 Liver fibrosis and primary hepatic stellate cell cultures

To date there are no approved direct anti-fibrotic agents for the management of liver fibrosis. Hepatic stellate cells are the major collagen producing cells during conditions of sustained hepatic injury (either metabolic, cholestatic, viral or toxic) leading to liver fibrosis, when hepatocyte damage triggers a cascade of events leading to activation of quiescent HSCs into a myofibroblastic (activated) HSC state. HSC activation is mediated by a plethora of pathways that finally result in increased secretion of extracellular matrix proteins, such as collagens, that accumulate as scar tissue (fibrosis) within the liver parenchyma and to liver cirrhosis in a later stage. Before HeMiBio/Seurat-1, the best *in vitro* fibrosis models consisted of mono-layer cultures of freshly isolated rodent HSCs in regular tissue culture dishes which leads to "spontaneous" HSC activation. Obvious limitations of these cultures are the rodent background and the un-controlled and hepatocyte damage-independent activation of the HSCs, making these cultures less suitable for pro- and anti-fibrotic compound testing translatable to human. During HeMiBio we developed a novel three-dimensional (3D) human co-culture model where both hepatocyte functionality and HSC quiescence can be maintained for at least 21 days. Unlike many currently used HSC cultures, the HSCs used are primary human and not cell lines. During HeMiBio, human HSCs from multiple donors were isolated and characterized at the genomic and methylome level as well as functional level. Optimisation and characterisation of human HSCs from multiple donors were used in these organoid cultures. This novel system allows hepatotoxicity testing as well as drug-provoked and hepatocyte-dependent HSC activation and fibrosis, a drug-induced liver injury (DILI) rarely addressed *in vitro* unlike steatosis, choleastatis and phospholipidosis. This is a big step forward from the regular 2D HSC cultures that are generally used and was published in Biomaterials (1$^{st}$ ranked material sciences, 2$^{nd}$ biomedical engineering) highlighting the broad impact of the paper. This work was presented at the SOT meeting in San Diego (2015) and at the EuroTox meeting in Porto (2015) and of course multiple times at the SEURAT-1 annual meeting. The hepatic organoid

work served as basis for a patent application (PCT/EP2015/062551).

These hepatic organoids represent a substantial improvement when screening for drug-induced liver fibrosis in terms of cost, animal use and prediction of liver fibrosis in human. Exploitation of these results comprises the provision of services for in-house measurements of fibrotic capacity of chemicals and potential screening of anti-fibrotics in specific fibrosis settings. Further development of more complex organoid cultures including also LSEC or even imflammatory cell types could expand the applicability of the organoid cultures (incl. clearance studies, infection etc.). Furthermore, this technology could stimulate the development of culture models representative of fibrosis in other organs such as lung and kidney, since these share common mechanisms.

## 4.4. Overall

The established AOP on cholestasis may have a considerable impact on the areas of toxicology and human risk assessment. Indeed, it may serve a number of purposes, such as the development of innovative *in vitro* and *in silico* tests, elaboration of prioritisation and tiered testing approaches, and chemical categorisation. Following generation of the cholestasis AOP, this conceptual tool has been tested for its predictivity, reliability and robustness in one of the SEURAT case studies conducted. The results confirmed the relevance of the existing elements of the AOP and identified potentially new ones, which in turn may lead to novel biomarkers of cholestatic injury. This AOP will be further tested in a number of recently started national and European research projects. This will contribute to the overall implementation of the 3Rs concept and thus will reduce the number of animal tests, which was a main driver of SEURAT.

Several toxicity assays have been optimised by the VUB-B team and therefore form the basis for the outcome of the project. Know-how associated with these assays might also be used to provide scientific and technical support to third parties, such as other research centres or private entities, in the framework of joint R&D projects or contractual research and consultancy services. Since the liver is the most important site of xenobiotic metabolism in the body, *in vitro* evaluation of metabolism and liver-related effects is an important stage in the development of new pharmaceuticals, cosmetics, chemicals and alike. Accordingly, companies operating in these sectors, as well as CROs performing toxicity testing, might be potential beneficiaries and adaptors of this particular foreground created within the HeMiBio project. With respect to commercial exploitation, no IPR protection will be possible or commercially viable, as all of the methodologies have already been described in scientific and technical literature. As such, they have only been adapted and optimised for the purpose of HeMiBio and other SEURAT projects.

The HeMiBio consortium has produced a plethora of scientific manuscripts, many related to the toxicity testing part of the project and published in high-impact journals, as will be outlined in detail in the second part of this report. Furthermore, the (toxicity testing) results of HeMiBio have been presented numerous times orally or by poster on international conferences and have been the subject of a number of awards. A cornerstone of the SEURAT project related to interaction between projects.

## 4.5. Training & Education

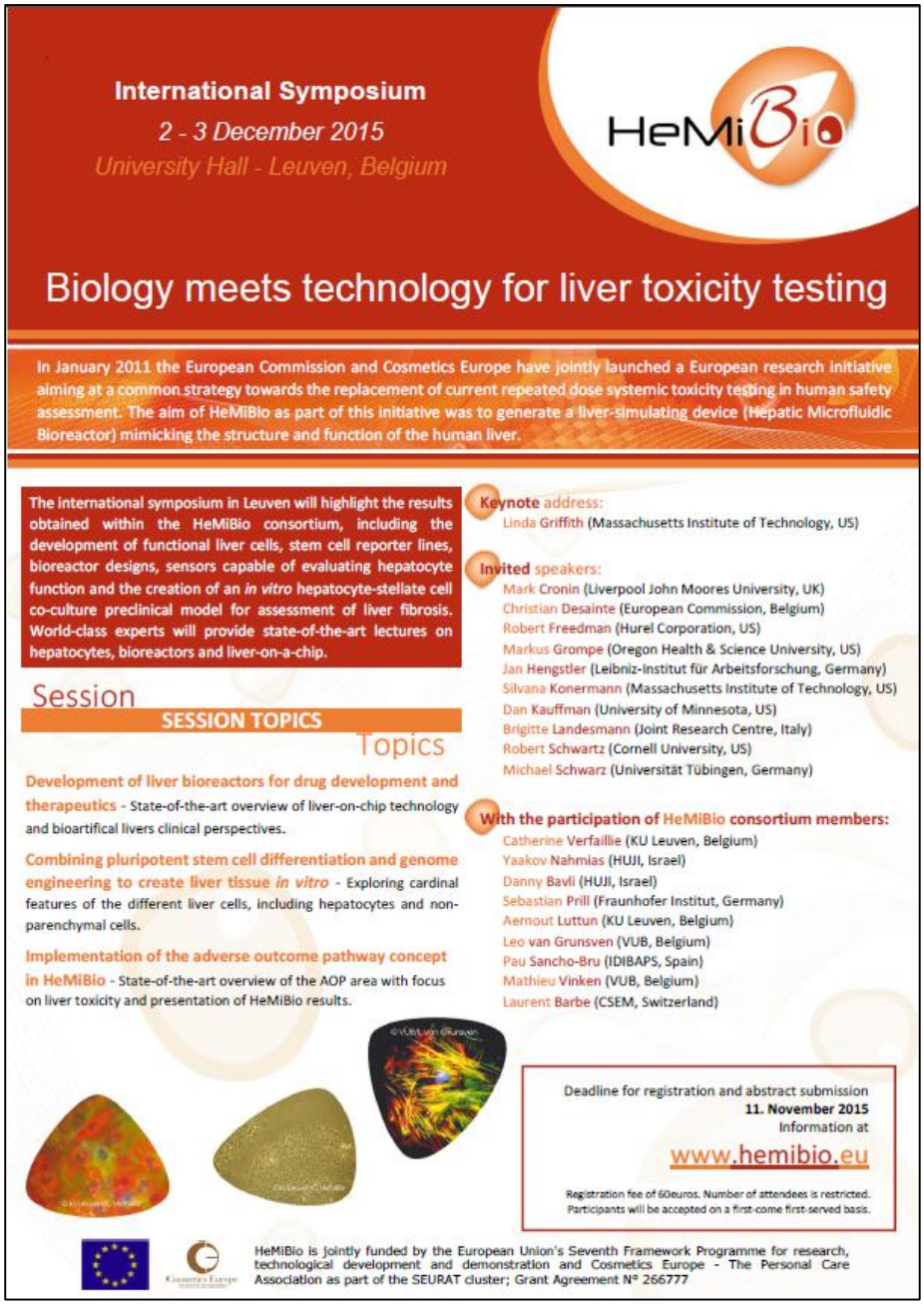
During the lifetime of the HeMiBio project the consortium has run an extensive Training and Education programme which included overall 4 Winterschools on HeMiBio related topics and 19 internal exchanges of young scientists allowing them to acquire knowledge in cross-disciplinary techniques. In addition HeMiBio supported participation of its young scientists at network relevant international congresses and workshops to foster their careers.

A highlight of this programme was the final meeting of the network which was organised as a public symposium on December 2$^{nd}$ – 3$^{rd}$ 2015 in Leuven, Belgium, bringing together renowned specialists in the field of liver biology, toxicology and engineering (**Figure 16**). The symposium served as a major dissemination event for HeMiBio relating the scientific advancements made over the last five years in the characterisation of human liver cells derived from either human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs) or transformed human cells. In addition tools and techniques were presented used to help understand toxicity mechanism and adverse outcome pathways (AOPs) of toxicity. The symposium highlighted the results

obtained within the HeMiBio consortium, including the development and differentiation of iPSC reporter lines and hepatic differentiation protocols, microfluidic bioreactor design and construction and development of sensors capable of evaluating hepatocyte function in repeated dose toxicity settings; and the creation of an *in vitro* hepatocyte-stellate cell co-culture model for assessment of liver fibrosis. In addition, sessions emphasized topics covered by other SEURAT-1 funded consortia, aside from state-of-the-art lectures by internationally renowned experts in these areas of research.

The following topics had been proposed: ***SESSION I:*** **Development of liver bioreactors for drug development and therapeutics** - The objective of the session was to give a state-of-the-art overview of liver-on-chip technology and to provide clinical perspectives on bioartifical liver projects.

***SESSION II:*** **Combining pluripotent stem cell differentiation and genome engineering to create liver tissue *in vitro*** - In this session the cardinal features of the different liver cells were described, including hepatocytes, but also the non-parenchymal cells, including hepatic stellate cells, hepatic sinusoidal endothelial cells, kupffer cells and liver resident innate immune cells.




***SESSION III:*** **Implementation of the adverse outcome pathway concept in** HeMiBio - The objective of the session was to give a state-of-the-art overview of the AOP area with focus on liver toxicity and to present the HeMiBio contribution.

Overall about 90 attendees were registered for the meeting. All participants had been invited to submit original posters. 23 posters were presented of which seven had been invited for short oral presentations of 10 minutes. Finally, two poster prizes were awarded chosen for their content and clarity by the HeMiBio Advisory Board members Daniel Duche (L'Oreal, Cosmetics Europe) and Philip Hewitt (Merck).

**Figure 16** Poster announcing the HeMiBio international symposium.

## 5. Partners involved and coordinator's contact details

| Part. # | Participant organisation legal name | Organisation short name | Principal investigator | Country |
|---|---|---|---|---|
| 1A | Katholieke Universiteit Leuven | KU Leuven | Catherine Verfaillie | Belgium |
| 1B | | | Aernout Luttun | |
| 2A | Vrije Universiteit Brussel | VUB | Leo van Grunsven | Belgium |
| 2B | | | Vera Rogiers/ Mathieu Vinken | |
| 3 | Universitetet i Oslo | UIO | Philippe Collas | Norway |
| 4 | Institut d'Investigacions Biomèdiques August Pi i Sunyer | IDIBAPS | Pau Sancho-Bru | Spain |
| 5 | The Hebrew University of Jerusalem | HUJI | Yaakov Nahmias | Israel |
| 6 | Interuniversitair Micro-Electronica Centrum VZW | IMEC | Jan Vanfleteren | Belgium |
| 7A | Fraunhofer-Gesellschaft zur Foerderung der angewandten Forschung E.V | Fraunhofer | Magnus Jäger/Claus Duschl | Germany |
| 7B | | | Marcus Heimann | Germany |
| 8 | Centre Suisse d'Electronique et Microtechnique SA – Recherche et Développement | CSEM | Silvia Generelli | Switzerland |
| 9 | Medicyte GmBH – *Project exit 30. June 2015* | Medicyte | Joris Braspenning | Germany |
| 10 | Medizinischen Hochschule Hannover – *Project exit 30. June 2012* | MHH | Toni Cathomen | Germany |
| 11 | Universitetet i Tromsoe | UIT | Bård Smedsrød | Norway |
| 12 | Inserm Transfert SA | IT | Christiane Dascher-Nadel | France |
| 13 | Universitaetsklinikum Freiburg - *Project entry 1. July 2012* | UKL-FR | Toni Cathomen | Germany |

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## 6. Project logo and public website

www.hemibio.eu