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New Models for the *In Vitro* Study of Liver Toxicity: 3D Culture Systems and the Role of Bioreactors

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1. Introduction

Present in all animal species, even if less developed in the lowest classes of the animal kingdom, the liver fulfils many vital functions of the utmost importance for the organism's survival. In adult humans, the biological significance of liver is attested by its anatomical localisation, its volume, its complexity, and by the density of the cellular elements it is composed of. Located in the right upper quadrant of the abdominal cavity, just below the diaphragm (Fig. 1), the liver represents, as a matter of fact, the largest visceral organ in the human body (about 2.5% of the dry body weight of an adult), and its parenchyma is constituted of more than 300 billion cells (Conti, 2005).

Physiological investigations have assigned to the liver a complex array of more than 500 different functions, the majority of which still remain unknown in their molecular mechanisms and controls. These functions include a number of key metabolic and regulatory activities, as well as processes crucial to the organism's defence (Arias *et al.*, 2009). The principal hepatic functions are schematised in Fig. 2. Indeed, besides carrying a central role in the metabolism of carbohydrates, lipids and proteins, the liver regulates other critical homeostatic functions, such as endocrine activity and haemostasis (synthesis/activation/catabolism of hormonal compounds, and of the majority of coagulation's/fibrinolysis' factors and inhibitors), and it directly acts as an integrant part of the systemic reaction to injury by, for example, modulating the immune response and synthesizing proteins from the "acute phase" (Nahmias *et al.*, 2006). In addition to its multiple metabolic activities, the liver also represents the first line of defence of the whole organism against exogenous or toxic substances. The liver is, in effect, the major site for inactivation of toxins and xenobiotics, favours their removal from the blood and further elimination from the organism through bile secretion (processes of biotransformation and excretion)[1] (Arias *et al.*, 2009).

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[1] **Biotransformation of drugs, xenobiotics, toxins and endogenous compounds.**
Part of lipophilic compounds can accumulate in the body (mainly in fat and bone tissues) or, alternatively, they need to be transformed in hydrophilic substances, in order to be more readily eliminated (excreted). Most of drugs are poorly hydrosoluble, and are metabolised (biotransformed) mainly at the hepatic level. Biotransformation process is the principal factor that can affect the overall

The importance of this organ and the impossibility to substitute artificially its multiple activities, justify, therefore, the serious clinical consequences of its dysfunction: until now, in industrialized countries, liver failure (acute and chronic) is among the top ten most frequent causes of death (Popovic & Kozak, 1998). A fundamental and typical feature of liver lies in the species-specificity of its functional characteristics and susceptibility to injury, elements that, both, make animal models commonly used for patho-physiological and pharmaco-toxicological studies, inadequate and poorly informative (or predictive) for humans (Rangarajan *et al.,* 2004; Sivaraman *et al.,* 2005). Examples are adverse drug reactions that occur in humans, which, being specifically based on liver metabolism or on hepatocellular sensitivity, are unpredictable or poorly understood (Park *et al.*, 2005).

To date, notwithstanding the long time period required (more than ten years), and the high financial investment necessary for the development of any new drug (estimated at around one billion Euros per molecule, of which, at least, one-fifth is used exclusively for toxicological investigations on hepatic function), almost two-thirds of the compounds that reach the phase III of clinical trials, demonstrate significant hepatotoxic effects in humans, which prevent their approval. Moreover, it is noteworthy that the hepatic toxicity (including idiosyncratic and chronic toxicity) unpredicted by the current experimental protocols represents more than one third of all the causes responsible for the withdrawal from the market of already approved drugs (O'Brien *et al.*, 2004; Whitebread *et al.*, 2005). Lastly, drug-induced hepatotoxicity is a major clinical pitfall, accounting for 50% of all cases of acute liver failure. From the above considerations, it is, therefore, clear how liver plays a unique and central role in toxicological studies: first, because it is critical in the pharmacokinetics of chemicals, due to its functions of biotransformation and excretion of substances, and, second, since it represent a foremost target of organ-specific adverse effects of drugs and xenobiotics.

In order to lessen and offset hepatotoxic effects, a large number of methods were developed and are currently applied in risk assessment procedures (*in vitro* and *in vivo* methods, human clinical trials, clinical case reports, etc.), or in toxicological/epidemiological studies (observation of the exposure-induced effects on human health), aimed to identify potential human hazards. Some of these methods are presented in Table 1, focusing on their features in relation to the fundamental characteristics required to fulfil the process of risk assessment.

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therapeutic and toxic profile of a drug; it can lead to detoxification, excretion, or, less frequently, to bioactivation of the chemical compound, being, thus, responsible for its biological activity, pharmacokinetics and clearance (Brandon *et al.,* 2003). Biotransformation occurs in three different phases: metabolic phases I and II, and transport phase III. The phase I reactions (functionalization step) include oxidation, reduction, or hydrolysis enzymatic reactions, that are, mainly, catalysed by the cytochrome P450 (CYP)-depending and flavin monooxygenase superfamily enzymes. Part of these phase I metabolites can be eliminated by biliary excretion, whereas another part can be metabolised by the phase II reactions, which allow to conjugate polar compounds (and metabolites) to water-soluble groups (glucuronic acid, sulphate, acetate, glycine, glutathione or methyl and acetyl groups), that render the derivatives much more soluble. Hydrophilic derivatives can then be excreted (mainly by kidney, even if liver excretion through bile also takes place). The third phase of compound biotransformation involves active membrane transporters, which, in hepatocytes, are located at their two polar surface domains. Apical and canalicular ATP-binding cassette (ABC) family of drug transporters are responsible for xenobiotic clearance (or bile secretion); basolateral solute carrier transporters, such as, for example, organic anion transporters (OATs), organic cation transporters (OCTs), and organic anion-transporting polypeptides (OATPs), are involved in the uptake of compound from the blood (Pauli-Magnus & Meier, 2003; Omiecinski *et al.,* 2010).

New Models for the *In Vitro* Study of Liver Toxicity:
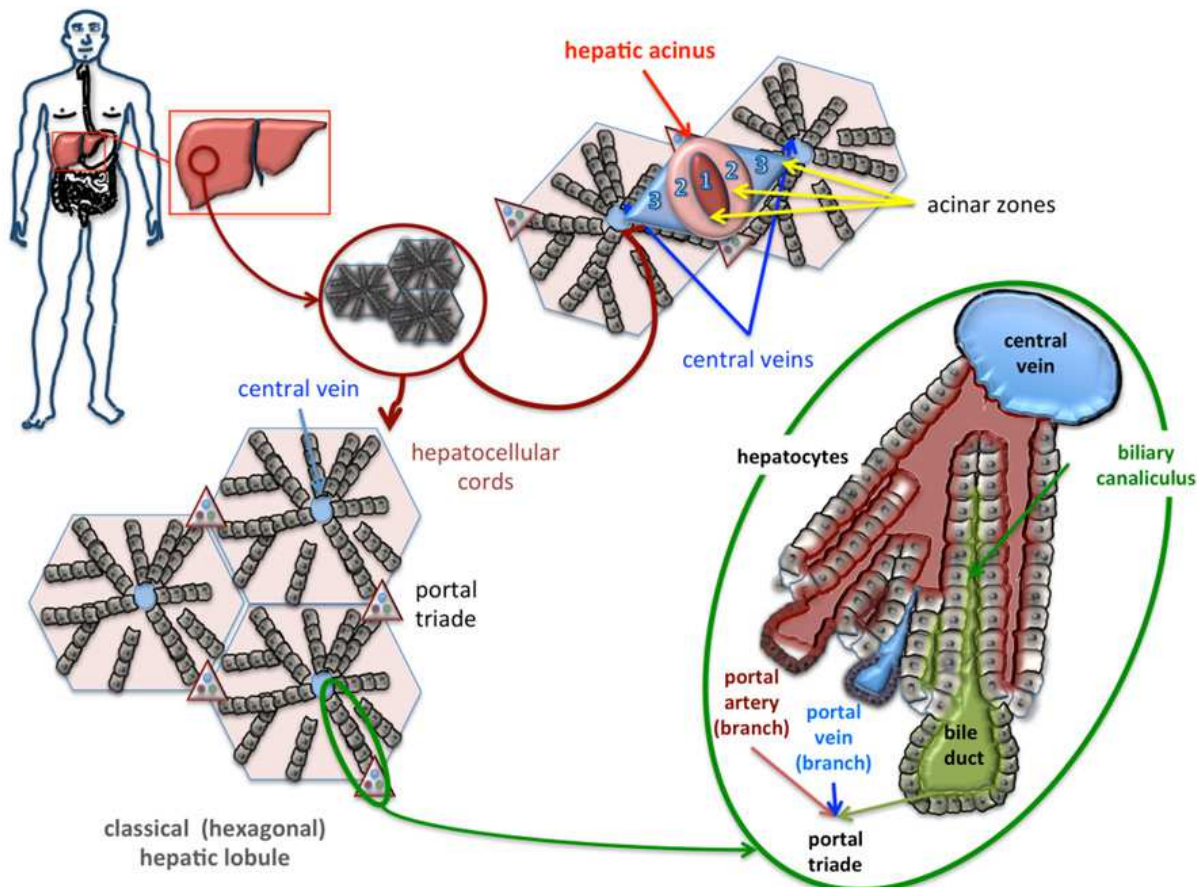3D Culture Systems and the Role of Bioreactors
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Fig. 1. Schematic view of the liver: location, macroscopical and microscopical organisation.

Since each model possesses advantages (and limits), the most promising strategy to assess the toxicological risk of substances and drugs, should be, at present, a combination of all the information obtained from these various models (integrated testing strategy). Table 2 lists the principal models currently used for hepatotoxicity studies.

Nevertheless, the predictive value for humans of current pre-clinical safety assessment systems is still limited and largely insufficient for a correct estimation of clinically relevant drug-drug interactions (DDIs) and pharmacodynamics/toxicological properties of compounds (ADMET profile) (Soars *et al.*, 2007). Significant inter-individual variability and great inter-species differences in liver functions, that make animal models inadequate for the safety testing of drugs and xenobiotics, have produced the necessity to develop new *in vitro* models, able to better reproduce or mimic the function of the human liver. In the last decades this necessity has already stimulated intense research activity, sustained by significant financial investments. The development of methods (and models) alternative to animal experimentation, along with the approval of their use, either in basic research, or in the more complex field of pharmaco-toxicology, has known a noteworthy expansion in the last twenty years[2] (see Table 2).

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[2] **"Alternative" methods and the 3R's principle.**
In 1959, Russel and Burch introduced, for the first time, the concept of methods "alternative" to experimental animal models (Russel & Burch, 1959). The authors defined as "alternative" any method that can be used in order to *Replace*, *Reduce* and *Refine* (3R's principle) the use of animals in biomedical
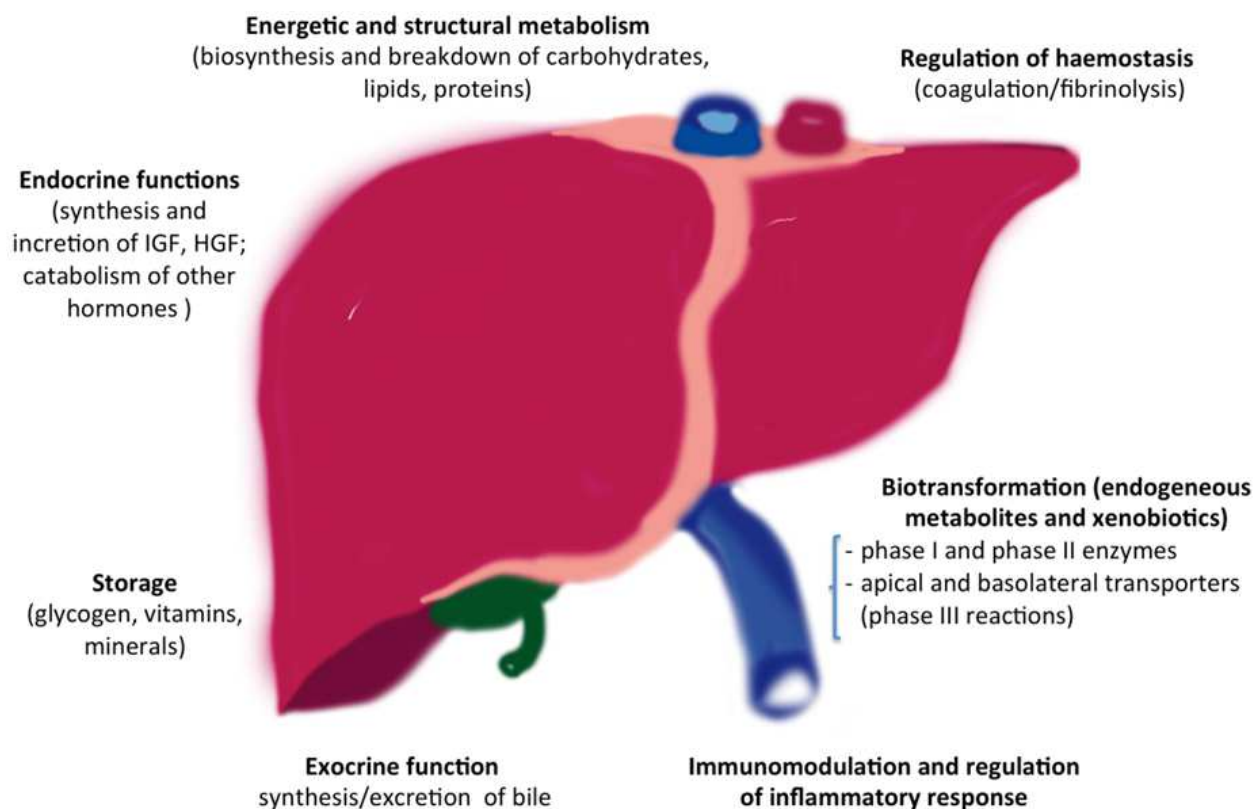
Energetic and structural metabolism
(biosynthesis and breakdown of carbohydrates,
lipids, proteins)

Regulation of haemostasis
(coagulation/fibrinolysis)

Endocrine functions
(synthesis and
incretion of IGF, HGF;
catabolism of other
hormones)

**Biotransformation (endogeneous
metabolites and xenobiotics)**
- phase I and phase II enzymes
- apical and basolateral transporters
(phase III reactions)

Storage
(glycogen, vitamins,
minerals)

Exocrine function
synthesis/excretion of bile

**Immunomodulation and regulation
of inflammatory response**

Fig. 2. Schematic representation of the principal liver's functions.

However, due to the high level of specialisation and complexity of the hepatic parenchyma, with its specific intra-lobular "zonal" organisation (Christoffles *et al.*, 1999), and given the peculiar sensibility of its cellular elements to even minimal environmental changes, none among the traditional and more widespread *in vitro* liver-derived models routinely available, seems to possess satisfactory features to be considered as a suitable model of the organ *in vivo* (Brandon *et al.*, 2003; Guillouzo & Guguen-Guillouzo, 2008). New approaches require, actually, the generation (and validation) of human-specific *in vitro* liver-derived test

research, testing or education. The basis of the 3R's principle is the aim to improve ethical standards and animal welfare in *in vivo* experimental procedures, by: i) **replacing**, as much as possible, *in vivo* models (use of alternative methods, i.e. *in vitro*, *ex vivo* and *in silico* approaches), ii) **reducing** the number of animals needed for experimental objectives, and iii) **refining** the experimental procedures, in order to reduce animal sufferance (pain, stress, discomfort). Until now, despite the great political and economical efforts that have been undertaken worldwide during the last two decades (especially by EU, US and other industrialised countries, such as Canada and Japan) to comply with the 3R's strategy (Mazzoleni and Steimberg, 2010; Hartung, 2010), only few alternative methods have been validated for regulatory toxicology and efficacy testing of chemicals. The Organization for Economic Co-Operation and Development (OECD), which represents 30 countries in the Americas (including the United States), Europe, and Asia, provides a collection of internationally harmonized testing methods for a number of toxicological endpoints using *in vivo*, *in vitro*, and even alternative approaches (OECD "Guidelines for the Testing of Chemicals"). For up-to-date information on the *Alternatives to Animal Testing*, see also "**Altweb** - the Alternatives to Animal Testing Web Site" http://altweb.jhsph.edu/), and, for specific bibliography, "**ALTBIB** - Resources on Alternatives to the Use of Live Vertebrates in Biomedical Research and Testing" at http://toxnet.nlm.nih.gov/altbib.html. On the web, **AltTox.org**, is a website dedicated to advancing non-animal methods of toxicity testing through online discussion and information exchange (http://alttox.org/).

Method Remark		*In vitro* (cell-based studies)	*In vivo* (animal-based studies)	Human clinical trials (volunteers)	Evidence-based toxicology, human exposure, epidemiology
Reliability of the predictive studies	single organ/tissue	+/-	+/-	++	+++
	whole human population: multifactorial aspects, sex, race age, healthy or pathological conditions	-	-	+/-	+++
Capability to mirror whole organism's response (systemic response, immune and hormonal regulation; all aspects of ADMET and DIDs) (*)		--	+++	+++	+++
Specific toxicity assessment (targeting liver-specific effects and "zonal" toxicity) (**)		-/+	++	++	++++
Range of lethal and non lethal endpoints (acute toxicity)		+++ (IC50)	++ (DL50 + LOEL)	--	Not applicable
Chronic toxicity assessment		-	++	+/-	+++
Wide-range investigations (time-/dose-dependent studies without inter-individual variability, repeated-dose effects, etc.)		+++	++	-	+++
Complex mixtures testing		+++	+++	-	+
Mechanistic studies		+++	++	+/-	+/- (macroscopic effect)
Reproducibility (control of experimental/testing conditions)		+++	+/-	+/-	-
Validation of the systems		+ (some endpoints)	+ +	Not applicable	Not applicable

Method ➡ ———————————————— Remark ⬇	*In vitro* (cell-based studies)	*In vivo* (animal-based studies)	Human clinical trials (volunteers)	Evidence-based toxicology, human exposure, epidemiology
Ethical features (3R's principle; environmental reduction of potentially toxic wastes, etc.)	+++	-	+	+++
Cost	+	++++	++++	++
General remarks	Validated models are still insufficient to be fully predictive for humans	Animal use, even if still necessary, should be reduced as much as possible	Should be performed after exhaustive pre-clinical studies on accurate/ validated models	By reducing pre-clinical, clinical and post-clinical toxicity, epidemiological studies could be reduced

(*) ADMET: absorption, distribution, metabolism, excretion, and toxicity; DIDs: drug-drug interactions.
(**) Zonal toxicity: specific peri-central/peri-portal toxic effects (Allen *et al.*, 2005).

Table 1. Comparison of the various liver models employed in hepatotoxicity studies.

model systems, which are able to give answers at the physiological level, and to be relevant to human outcomes. While consistent evidence demonstrates the breaking points of traditional *in vitro* models (static culture in monolayer) in reproducing the behaviour and physiological response of various tissues (the hepatic one, in particular), contemporarily, three-dimensional (3D) systems are achieving an increasing status (Mazzoleni *et al.*, 2009). In effect, the more promising results seem, at present, to derive from the 3D techniques of culture, that, by guaranteeing the preservation of at least some characteristics of the complex hepatic microenvironment, can favour cell survival and the *in vitro* expression of the liver-specific differentiated phenotype, allowing, in such a way, the generation of more reliable and predictive hepatic test models for human investigations (Shvartsman *et al.*, 2009).

In the present chapter, the principal models available for the *in vitro* study of liver functions are reviewed. Moreover, the contribution of new emerging technologies and tissue engineering, as basis for the conception of innovative hepatic models, the relevance of 3D bio-constructs, as reliable liver analogues, and their relative advantages and drawbacks in the process of being developed/validated, is discussed. A particular attention is given to the model systems based on the use of dynamic bioreactors, and, more specifically, on the use of the *Rotary Cell Culture System* (RCCS™, Synthecon, Inc.) device.

2. Currently available liver-derived *in vitro* models and the role of liver-specific microenvironment

The need for studying hepatic functions, and for elucidating the molecular mechanisms at their basis, has resulted, over time, in the development of a huge number of liver-derived models and systems, that has not been equalled in the case of any other human organ/tissue (see Table 2).

Models	Advantages	Drawbacks	References
Human liver-derived single enzymes, supersomes, sub-cellular and organelles' fractions (cytosol, microsomes, mitochondria, S-9 fractions)	- Useful for identifying mechanisms/enzymes/ metabolism of drug- and chemical-induced hepatotoxicity - Contain a rich variety of metabolic enzymes for studying the *in vitro* metabolism of drugs - High availability - Easy to prepare, use and store - Mitochondrial dysfunctions and energy metabolism studies are allowed - Molecular/sub-cellular investigations	- Only sub-acute/acute toxicity studies - Extremely simplified models, lacking whole gene regulation systems, cell-cell interactions, bile canaliculi, transporters, cell structures and functions - For extrapolation to whole cell, all sub-cellular fractions should be tested - Low level of enzyme activity may be faced - Difficult extrapolation to whole *in vivo* organism - Lack of concordance with the *in vivo* situation (microsomes lack some phase II enzymes)	Boelsterli & Lim, 2007 Clarke & Jeffrey, 2001 Guengerich, 1996 Mae *et al.*, 2000 Rawden *et al.*, 2005
Reporter gene-based systems (mainly P450 enzymes-expressing models): recombinant hepatoma cells, liver supersomes, microsomes	- Supply drug-metabolizing or functional capacity to cellular systems lacking metabolic enzymes/ transporters - Provide information about single CYP enzymes - Readily available - All known human cytochrome P450 (CYPs) have been successfully over-expressed in genetically modified organisms	- Only sub-acute/acute toxicology studies - Lack of other phase I and phase II biotransformation enzymes - Extremely simplified model - The transduced isoform is in excess as compared to physiological concentration *in vivo*	Huang *et al.*, 2000
Single liver cells analysis	- Single cell approach of biological process - High availability of cells - Possibility to test high broad number of drugs in various culture conditions	- Very short-term studies - Sub-acute/acute toxicity studies - Need optimisation of investigation tools	O'Brien *et al.*, 2006 Xu *et al.*, 2004
Isolated hepatocytes in suspension	- Highly available - Easy-to-handle - Poor functional activity - Applicability to High-throughput screening (HTS): limited - Could support kinetic, and drug-drug interactions studies	- Short-term cell viability (2-4 hrs) - Sub-acute/acute toxicity studies - Phenotypic instability due to alteration of tissue architecture and ECM, loss of cell polarisation/organisation - Loss of cell-cell / cell-ECM interactions - High variability between batches - Absence of non-parenchymal cells	Guillouzo, 1998 Richert *et al.*, 2006
Hepatic cell lines in conventional 2D culture (examples of human-derived cell lines: HepG2, Hep3B, HepaRG, HepZ, C3A,THLE; examples of non-human hepatic cell lines: HTC, BRL3A and NRL clone 9, Fa32 and WIF-B9)	- Almost infinite capacity of proliferation - High availability - Easy-to-handle (culture, freezing, etc.) - Possibility to genetically engineer cells (Hep3B, HepaR, THLE) - Avoid repeated cell isolation - High reproducibility - Moderate cost - Applicable to HTS - Differentiated phenotype variably expressed, according to the cell line (e.g. HepG2 cells express CYP1A,	- Acute toxicity studies - Lack or limited/partial drug-metabolism capacity and other hepatic functions - Absence of non-parenchymal cells - Expression of typical phenotype, depending on culture conditions and passaging - No accurate modelling of *in vivo* hepatic phenotype (genetically instable and/or derived from malignant tissues)	Boess *et al.*, 2003 Dierickx, 2003 Guillouzo *et al.*, 2007 Jennen *et al.*, 2010 Kanebratt & Andersson, 2008 Malatesta *et al.*, 2008 Payen *et al.*, 1999

Models	Advantages	Drawbacks	References
	CYP2B, CYP3A, CYP2E, UDPGT, GST, but lack other drug metabolizing enzymes; HepRG cells express CYP1A2, CYP2B6, CYP2C9, CYP3A4, CYP2E1, GSTAs, UGT1A1, some ABC transporters, some plasma proteins, but not always in homogeneous way) - According to the cell line: metabolic profiling; kinetic studies; drug-drug interaction, mechanistic studies and short-term toxicity screening		Richert, *et al.*, 2006 Schoonen *et al.*, 2009 Slany *et al.*, 2010 Werner *et al.*, 2000 Wilkening & Bader, 2003
2D primary cultures of hepatocytes in static conditions (monotypic cultures)	- Primary human hepatocytes represent the *in vitro* model of choice for drug screening - Express most of CYP isoforms (e.g. CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4) - Express phase II biotransformation enzymes (e.g. UDPGT, SULT2A1, GST) - Easy to use and fast - Short-term preservation of cell viability, liver-specific functions and gene responsiveness - High/medium efficient cryopreservation - At high density or confluence, cell-cell contacts may be rebuilt - Reproducible method - Short-time inducibility of phase I and phase II enzymes by xenobiotics - Applicable to HTS - Could support: metabolic profiling, pharmacokinetic (ADME) and drug-drug interactions (DIDs) studies, mechanistic studies and short-term toxicity screening	- Early phenotypic alterations (about 75% of total CYP activity within the first 24 hrs)= loss of accuracy - Scarcity of human liver - Isolation is time consuming and may damage cells - Limited proliferation - Difficulty to mimic the *in vivo* microenvironment - Loss of cell polarity, tissue architecture, membrane domains - Lack of non-parenchymal cell types - Inter-donor variability (human liver) - Batch-to-batch variability of isolated hepatocytes - High influence of culture conditions on cell features - Inter-species variability	Gomez-Lechon *et al.*, 2007 Guillouzo, 1998 Hewitt *et al.*, 2007 Lecluyse *et al.*, 2001
Somatic cells (trans-differentiation into hepatocytes), hepatic stem/ progenitor cells	- Could express a large panel of liver-specific genes (including those involved in biotransformation processes)	- A great effort is needed to reach a fully differentiated phenotype and for use in risk assessment - Variability in the phenotype expressed by "hepatocytes"	Lee *et al.*, 2004 Schwartz *et al.*, 2002
2D co-cultures of liver-derived cell types (heterotypic culture, at large- or at micro-scale)	- Higher cell viability than monotypic culture (2 weeks) - Maintain some hepatic functions for longer time (plasma protein, urea secretion, lipoprotein metabolism, some CYPs) - Maintain heterotypic cell-cell interactions	- Only some liver specific functions are slightly maintained after longer time in culture (up from 7 to 15 days)	Guzzardi *et al.*, 2009 Ijima *et al.*, 2005 Khetani & Bhatia, 2008 Ohno *et al.*, 2008
3D cultures of primary hepatocytes/hepatic cell lines/stem cells without (spheroids,	- Maintain more hepatic functions for longer time (up to some weeks) - A number of genes are up-regulated (albumin, transferrin, fibrinogen,	- Cell recovery is sometimes difficult - Loss of liver-specific functions (decline of some CYP activities) - In some 3D models, formation of	Chang & Hughes-Fulford, 2009 Du *et al.*, 2008

Models	Advantages	Drawbacks	References
micromasses) or with scaffolds (micropatterned polydimethylsiloxane surfaces; nanofibrillar network; hydrogels Sandwich cultures: collagen-collagen, collagen-matrigel, alginate encapsulation, or RGD-galactose)	prothrombin, CYP1A1, CYP1A2, CYP2E1, CYP3A, etc.) - Ureagenesis varies according to the model, and may be 6-7 times as in classical 2D culture - High level of hepatic transporters - Fluidic dynamic of some bioreactor favours shear stress and support liver-specific gene expression - Improve microenvironment features - Hepatocytes are much more sensitive to some xenobiotics than in static 2D culture - After optimisation of culture and microenvironment conditions, these models could be used for chronic toxicology	necrotic cores: - According to the 3D model, specific hepatic function are maintained - Currently unsuitable for HTS (need the development of adequate methods/devices for increasing sensitivity and reliability, lowering cost and time-consuming features)	Evenou *et al.*, 2007 Kienhuis *et al.*, 2007 Liu Tsang *et al.*, 2007 Maguire *et al.*, 2007 Meng, 2010 Miranda *et al.*, 2010 Suzuki *et al.*, 2008 Walker & Woodrooffe, 2001
3D co-cultures of liver-derived cell types	- Longer cell viability, as compared to monotypic 3D culture (up to 57 days) - Sustain some liver-specific function from 3 days to 7 up weeks (albumin, urea secretion, expression of CYP1A1/2,CYP2B1, CYP3A) - Mimic liver cyto-organisation/cyto-orientation - Intercellular interactions and communications - Soluble factors enhance hepatocytes functions	According to culture conditions: - variable cell viability and differentiation status - need bioreactor optimisation to increase mass transfer - *in situ* approaches/techniques need to be developed to be applicable for HTS (see 3D monotypic cultures)	Bennett *et al.*, 2006 Bhatia *et al.*, 1999 Cheng *et al.*, 2008 Leite *et al.*, 2011 Ohno *et al.*, 2008 Riccalton-Banks *et al.*, 2003
Precision-cut liver slices	- Several aspects of *in vivo* microenvironment are preserved - Retain *in vivo* cyto-/histo-architecture - Acinar sub-localization of functions - Cellular heterogeneity (include non-parenchymal cells) - Expression of functional drug metabolizing enzymes (CYP1A, CYP2A, CYP2B, CYP2C and CYP 3A sub-families) and transporters - Preservation of hepatocyte polarity - Could support metabolic profiling, mechanistic studies and, less easily, toxicity screening	- Short-term viability (about 5 days) - Short-term metabolic studies (about 48 to 72 hrs) - Progressive formation of necrotic cores - Poorly amenable to HTS - Limited liver-specific functions - Scarcity of human liver donors - High intra-assay variability - Donor–to-donor variability - Hard to handle - Poorly efficient cryopreservation - Poor diffusion of drugs across the slides	Elferink *et al.*, 2008 Krumdieck, *et al.*, 1980 Lake *et al.*, 1996 Schumacher *et al.*, 2007
Isolated perfused liver (resections/whole animal liver)	- Maintenance of whole organ features and functionality (cell-cell and cell-ECM interactions, cell polarisation, cell heterogeneity, 3D organisation, zonation) - Represent the closest models mimicking *in vivo* situation - Allow real-time bile collection/analysis and oxygen consumption - Liver injury is reflected by LDH, AST, and ALT	- Very short-term studies are possible (2-3 hours) - Hard to handle - Expensive model - Poor reproducibility - Difficult inter-species extrapolation - Impossibility to be applied to human liver	Gores *et al.*, 1986 şahin, 2003

Models	Advantages	Drawbacks	References
Integrated discrete multiple organ co-culture systems	- Try to re-create the organ-organ cross talks (paracrine factors) - Assessment of organ-specific toxicity - Short-term (48 hrs) assessment of the biological/toxicological effect of native drugs and their metabolites/catabolites	- Need optimisation for HTS - Biotransformation of drug need to be known earlier to optimise cell culture and spatial organisation (or determined by random positioning) - Some limitations related to the 2D configuration	Li, 2008 and 2009
Whole organism	- Maintenance of systemic interrelations, tissue integrity and normal hepatic functions (for the considered specie) - Maintenance of liver physiology - Well known and often standardized methods - Whole system inter-relation is preserved - Allow to take into account the biokinetic features of drugs/molecules, (ADME/toxicology processes are maintained)	- Ethically discussable - Need to be revised for applying the 3R's concept - Need of GPL practice - Inter-species variability and difficulty to reflect human context (human-specific metabolites, inherent sensitivity of peculiar population) = difficulty and risk of unreliability, extrapolation of results from animal to human for xenobiotic metabolism as well as target organ sensibility - In clinical trials: inter-individual variability, limitations of trial endpoint and population sampling - Expensive and time consuming - Uncontrolled sources of variability, such as housing conditions of animals, subjectivity in scoring, etc.	Li, 2004

New computational *(in silico)* models, toxicogenomics-, transcriptomics-, proteomics- and metabolomics-based models present important advantages for risk assessment, but they need adequate *in vivo* or *in vitro* models to be reliable (Cheng & Dixon, 2003; Khor *et al.,* 2006; Hunt *et al.,* 2007; Valerio, 2009; Amacher, 2010; Gómez-Lechón, *et al.,* 2010).

Table 2. Experimental approaches used for hepatic risk assessment.

The first and simplest *in vitro* liver models were optimised for studying single metabolic functions, and are based on the use of hepatic sub-cellular fractions (single enzymes, microsomes, supersomes, cytosolic fractions and mixed fractions). Although such models are easy to use, they fail in mimicking the complete and complex metabolic potential of hepatocytes, as well as intra-lobular zonal specialisation and inter-individual heterogeneity of the liver-specific cell phenotype. Not even the models based on the use of liver-derived cell lines are considered to be fully reliable, even though, for their metabolic characteristics, they are more complete than the sub-cellular fractions, and present the advantage of limitless culture time. These cell lines, generated from malignant tumours or obtained from transformed cells, present, in effect, the disadvantage of having lost the majority of their original phenotypic features.

In the same way, also engineered cells or systems (e.g. microsomes) based on reporter gene transfer have been shown to be not sufficiently reliable and informative (for more exhaustive information on the principal *in vitro* hepatic models traditionally in use, see also Brandon *et al.,* 2003, Zucco *et al.,* 2004, Gómez-Lechón *et al.,* 2007, Guillouzo & Guguen-Guillouzo, 2008).

It is now well recognised that any experimental model that has to be used for reproducing *in vitro* the function of human liver, must be developed from human hepatocytes in primary culture (Gómez-Lechón *et al.,* 2007). The scarce availability of tissue, its variable quality, and the difficulty to succeed, with the traditional techniques, in maintaining isolated hepatocytes *in vitro*, preserving their viability and functions for long-term studies, have strongly hindered the refinement of these models, limiting, in such a way, the significance of their use (Gómez-Lechón *et al.,* 2007; Guillouzo & Guguen-Guillouzo, 2008). No result worthy of further consideration has been derived from the very large number of attempts performed to get well-differentiated hepatocytes from stem precursors, either originating from the liver itself (resident hepatic progenitors), or from extra-hepatic sites (e.g. bone marrow and adipose tissue), or from mesenchymal cells, obtained in precocious phases of the development (cells from umbilical cord blood or embryonic stem cells) (Cantz *et al.,* 2008). Advantages and limits of these cells as "hepatocyte donors" are summarized in Table 3.

Cell type	Origin	Characteristics	Reference
Embryonic stem cells (ESCs)	Derived from the inner cell mass of pre-implantation-stage blastocysts	⊕ Totipotent stem cells ⊕ Self-renewal ⊕ Huge proliferative potential ⊕ High differentiation potential and plasticity ⊕ Expression of early markers of hepatic differentiation ⊕ Some mature hepatic functions are maintained ⊖ Difficulty to be regulated and maintained under controlled conditions ⊖ Risk of oncogenicity ⊖ Ethical issues ⊖ Limited availability	Agarwal *et al.*, 2008 Baharvand *et al.*, 2006 & 2008 Ishii, *et al.* 2010 Jozefczuk *et al.*, 2011 Liu T *et al.*, 2010 Rambhatla , *et al.*, 2003 Soto-Gutierrez *et al.*, 2007
Extra-hepatic adult stem cells	Bone marrow	⊕ Pluripotent stem cells ⊕ Inducible to express a number of liver-specific functions ⊖ Difficulty to reach full adult hepatocyte phenotype ⊖ Less differentiated than primary hepatocytes	Avital *et al.*, 2001 Chen *et al.*, 2006 Chivu *et al.*, 2009 Petersen *et al.*, 1999
	Adipose tissue	⊕ Pluripotent stem cells ⊕ Express to some extent specific hepatic functions ⊖ Quantitatively less differentiated than HEPG$_2$ cell line ⊖ Less differentiated than primary hepatocytes	Okura *et al.*, 2010
	Umbilical cord blood	⊕ Pluripotent stem cells ⊕ Express some hepatic markers ⊖ Further investigations are necessary to better characterize their differentiation status ⊖ Less differentiated than primary hepatocytes	Campard *et al.*, 2008 Hong *et al.*, 2005 Lee *et al.*, 2004
	Peripheral blood	⊕ CD14+ peripheral blood monocytes ⊕ Well differentiated hepatocyte-like cells (expression of a number of liver-specific functions) ⊕ Easy to obtain ⊖ Multi-laboratory investigations should be done to validate the method	Ruhnke *et al.*, 2005 Ehnert , 2008 and 2011

Cell type	Origin	Characteristics	Reference
Adult/foetal stem cells and hepatic progenitors	Liver	⊕ Multi-potent stem cells ⊕ Different adult stem cells typologies ⊖ Still difficult to use for therapeutic applications ⊖ Hardly obtainable	Dan & Yeoh, 2008 Turner, 2011
	Hepatic progenitors (usually localised in small biliary canals)	⊕ Bi-potential cells ⊕ Differentiate in both hepatocytic and biliary lineages ⊖ Low cell number (in adult liver) ⊖ Difficult proposal for human derived cells	Dan & Yeoh, 2008 Zhang et al., 2008
	Foetal hepatocytes, hepatoblast precursors	⊕ Multi-potent stem cells ⊕ High proliferation rate ⊕ Exhibit some biotransformation pathways ⊕ Bi-potential plasticity ⊖ Ethical problems for human donors	Dan et al., 2006, Ring et al., 2010
	Somatic adult cells: hepatocytes	⊕ Temporary high differentiated phenotype ⊖ Unipotent cells ⊖ Low replication rate ⊖ Rapid cell function decrease ⊖ Low cell availability for human cells	Gomez-Lechon et al., 2007
Induced pluripotent stem cells (iPS)	From connective tissues (fibroblasts or other somatic cells)	⊕ Pluripotent cells ⊕ Highly proliferative cells ⊕ Capability to differentiate into liver-specific parenchymal cells ⊕ Quite similar to hESCs ⊕ Very promising technology ⊖ Lowest replication and differentiation capability than ESCs ⊖ Immature and incomplete hepatic phenotype ⊖ Still need the optimisation of differentiating protocols ⊖ Development of iPS without virus could represent a great challenge also for therapeutic applications ⊖ Need further development of differentiating strategy (type of differentiating factor to be used, spatio-temporal release of factors), optimisation and standardisation of induction protocols and culture conditions, as well as an increase of the quality control of the final cell preparation	Takahashi et al., 2007 Espejel et al., 2010 Hu et al., 2010 Q. Feng et al., 2010 Sullivan et al., 2010 H. Liu et al., 2010 & 2011 Greenbaum, 2010 Si-Tayeb et al., 2010
Trans-differentiated cells	Epithelial cells	⊕ Bi-potent cells ⊖ Immature cell functions are expressed	Snykers, 2007
	Pancreatic cells (healthy or tumour-derived)	⊕ Some differentiated hepatic enzymes are expressed ⊖ Difficult to propose for human-derived cells	Tosh , 2002 Burke et al., 2006 and 2007

Table 3. Cell types commonly used for the design of *in vitro* hepatic models and liver tissue engineering.

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The hepatocyte is a structurally complex epithelial cell and represents the major hepatic parenchymal cell type, either in terms of mass (60% of the total number of liver cells and 80% of the total volume of the organ), or for the number of functions carried out. Cholangiocytes and non-parenchymal cells [sinusoidal endothelial cells, stellate cells (fat-storing Ito cells), pit cells (intrahepatic lymphocytes), Kupffer cells, and hepatocyte precursors] influence, by their presence and activity, hepatocyte survival and function (Selden *et al.,* 1999; Riccalton-Banks *et al.,* 2003; Nahmias *et al.,* 2006; Catapano & Gerlach, 2007). The extracellular matrix (ECM) represents another key component of this organ: it is peculiar by composition and structure, and it plays a determinant role in regulating hepatocyte viability, as well as other biological processes, such as development, proliferation, migration and functional activity (Selden *et al.,* 1999; Van de Bovenkamp *et al.,* 2007). Being functionally and structurally polarised, the hepatocyte requires, in effect, precise and specific cell-cell and cell-matrix interactions. The characteristic cyto-architecture of liver, organised as "lobular" units, entails, furthermore, a particular morphological and functional specialisation of hepatocytes, that varies with position along the liver sinusoid, from the portal triad to the central vein ("zonation") (see Fig. 1 and Fig. 3). It is now well accepted that this regional compartmentalisation of metabolic (and detoxification) functions of hepatocytes within the "acinus" is responsible for the "zone-specific" liver susceptibility to many hepatotoxic agents (Lindros *et al.,* 1997). The hepatocellular "zonation", sustained by portocentral patterns of gene expression ("gradient" *versus* "compartmental" and "dynamic" *versus* "stable" types of zonation), is modulated by chemical gradients of oxygen, hormones, growth factors and metabolites, which are generated and maintained as a result of the specific characteristics of the ECM and of the distribution of non-parenchymal cells (Gebhardt, 1992; Christoffels *et al.,* 1999). This specific microenvironment, dynamic, highly organised and rigidly structured, is thus fundamental for maintaining hepatic functions. It is, actually, well known, how the subversion of liver architecture produced by the alteration/destruction of the strict relationship hepatocyte-matrix and hepatocyte-non parenchymal cells, that occurs as a consequence of trauma or pathological states (e.g. fibrosis), is responsible for important negative effects on liver homeostasis and loss of functions (Selden *et al.,* 1999; Van de Bovenkamp *et al.,* 2007). Experimental models of liver should, therefore, take into account the strict inter-dependence between the complex histo-morphology of this organ and its functions/responses (Allen *et al.,* 2005).

The need to preserve the original cytological and histo-architectural features of the hepatic tissue has provided the basis for attempting to develop various *ex vivo* hepatic models (Table 2). Among these models, the best known is the isolated and perfused organ model (Gores *et al.,* 1986, Bessems *et al.,* 2006). Although this model is considered to be the closest representation of the *in vivo* situation, its use is limited to a few hours; moreover, the necessity to employ a whole organ for each single experimental point, leads to a very broad range of variability in the results (Gores *et al.,* 1986), and makes impossible the use of human-derived tissue. As an alternative, the model based on the culture of organ slices, obtained by particular section methods (precision-cut liver slices) has been developed. Notwithstanding this model may solve, at least partially, the problem of results' variability, it still presents, employed with the conventional culture techniques, the disadvantage of limited cells' survival (only a few days), even if slice thickness is maintained under the physical limit of diffusion of gas and nutrients (200 µm) (Fisher *et al.,* 2001, Vickers *et al.,* 2011). Despite the development of new dynamic culture methods (see later) renders the
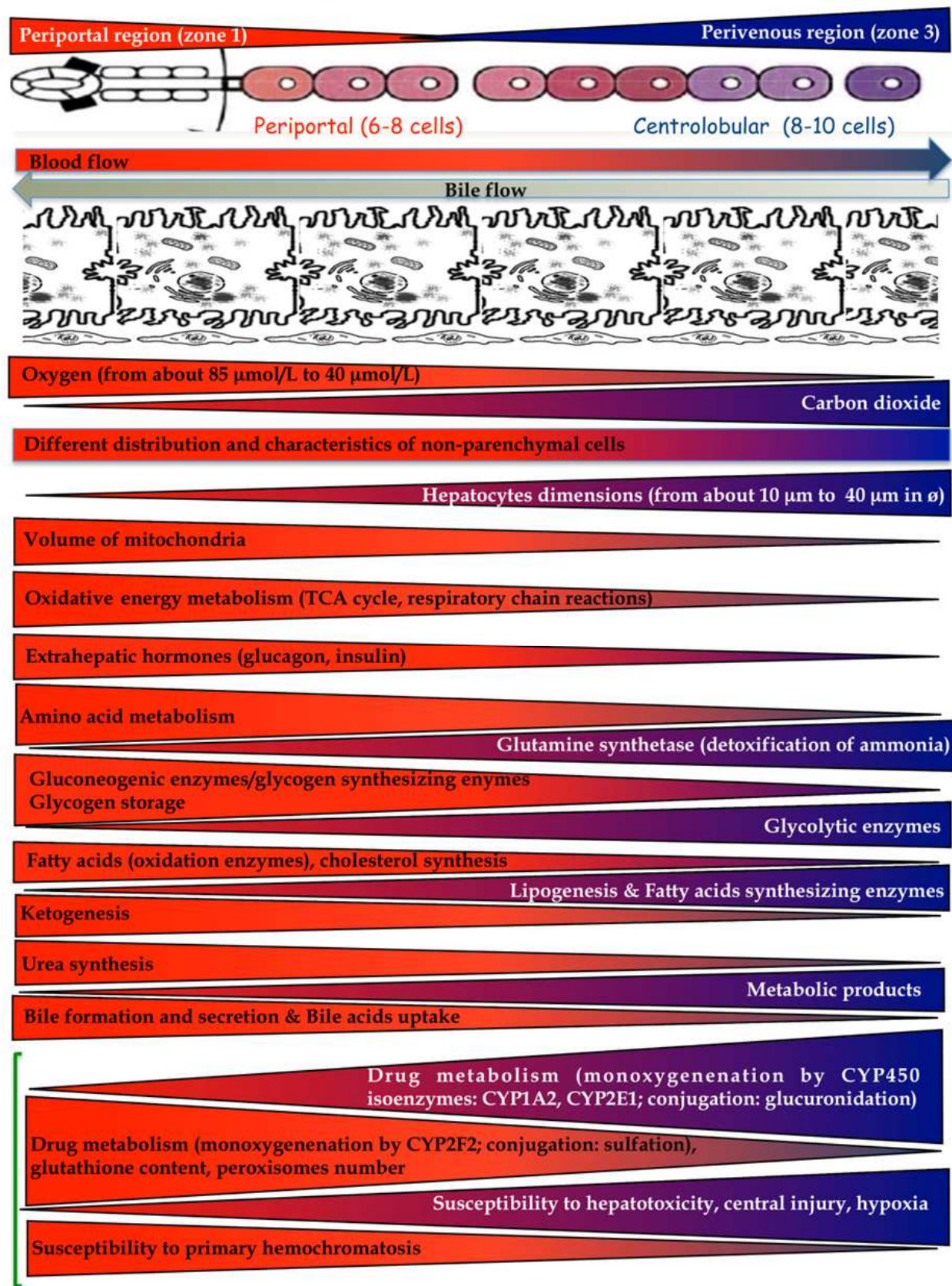
Fig. 3. Zonal specialisation of liver acinus, from periportal to centrolobular region, and different susceptibility to toxic agents.

future of this model very promising, due to the fact that, by preserving intact the original tissue microenvironment (ECM, multi-cellularity and histo-architecture), it could be very close to the liver *in vivo* (Van de Bovenkamp *et al.*, 2007), liver slices do not possess, at present, such characteristics. The more ordinary models used in the study of hepatic function, with their relative advantages/disadvantages, are illustrated in Table 2.

3. Three-dimensional liver-derived *in vitro* systems: Tissue engineering and the contribute of the new technologies

As in the case of other tissue models, it is now generally accepted that any attempt aimed at the generation of reliable and physiologically relevant *in vitro* liver analogues, should take into account the need of reproducing (or conserving) the specific characteristics of the original microenvironment typical of that organ (see above). The main features of the environmental context within which cells physiologically grow, proliferate and express their own functions, include, in addition to multiple cellularity, biochemical and mechanical properties (that are specific of each organ/tissue), also the three-dimensionality (Mazzoleni *et al.*, 2009).

Over the last few decades, it has already been widely demonstrated that, compared to the use of traditional culture techniques in monolayer (2D), three-dimensional (3D) culture methods allow researchers to generate *in vitro* tissue-derived model systems that better mimic the *in vivo* situation (Pampaloni *et al.*, 2007). Significant differences have, in fact, been demonstrated between the biological behaviour of cellular elements (hepatocytes included) maintained in culture with traditional (2D) culture methods, and that of cells kept in 3D culture (Mazzoleni *et al.*, 2009). Figure 4 presents a qualitative comparison between the most important characteristics of the different currently used liver model systems.

The importance of being able to reproduce *in vitro* the 3D specific microenvironment typical of the tissue of origin, has led to the design and development of increasingly complex and sophisticated 3D culture methods. These methods, benefiting also from the rapid development of tissue engineering techniques, have produced, especially in the case of the liver, an extremely wide variety of models (Nahmias *et al.*, 2006). In the case of the liver, due to the high structural complexity of its tissue microenvironment (see above), any attempt to apply the principles of tissue engineering aimed to generate constructs capable of reproducing, *in vitro*, the specific characteristics of the organ *in vivo*, implies to face extremely difficult technical problems, which also result from having to consider issues that must be performed on spatial and temporal scales that are "gigantic". For example: the hepatocyte can recognize the structural characteristics of the surface on which it must adhere with a threshold of nanometers in size, but it should organise itself in hierarchical structures of centimetres in size; similarly, the presence of particular molecules may alter the structural characteristics of the hepatocyte's microenvironment within a few milliseconds, while the time required for the cell to functionally adapt to these changes can take several weeks (Mitzner *et al.*, 2001).

Over the years, the research in this direction has been mainly devoted to the generation of new materials (micro- and nano-structured), which possess physical and biochemical characteristics suitable to fulfil mechanical and biological support to the physiological hepatocyte activities. In particular, also due to the contribution of the new emerging technologies, the number of liver models that have been generated within the last ten years, by using the principles of tissue engineering, has been enormous.
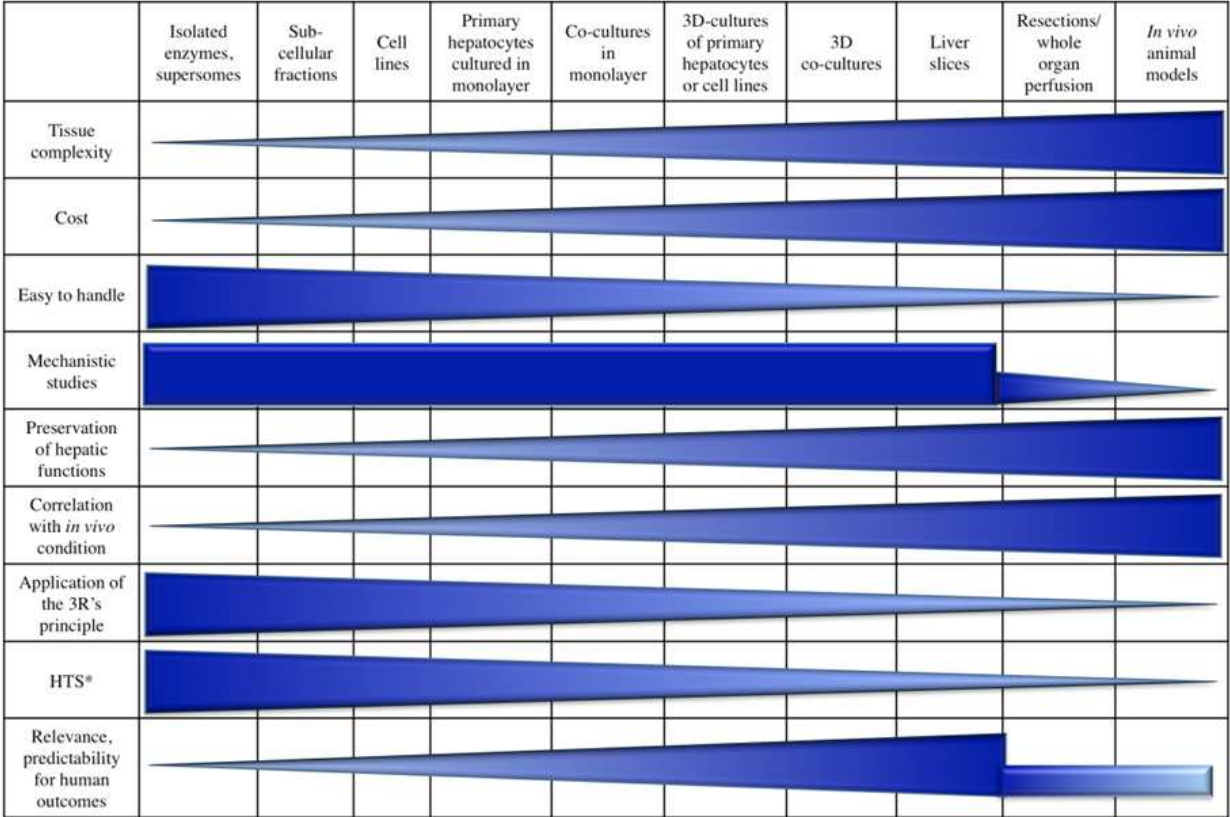
	Isolated enzymes, supersomes	Sub-cellular fractions	Cell lines	Primary hepatocytes cultured in monolayer	Co-cultures in monolayer	3D-cultures of primary hepatocytes or cell lines	3D co-cultures	Liver slices	Resections/ whole organ perfusion	*In vivo* animal models
Tissue complexity										
Cost										
Easy to handle										
Mechanistic studies										
Preservation of hepatic functions										
Correlation with *in vivo* condition										
Application of the 3R's principle										
HTS*										
Relevance, predictability for human outcomes										

Fig. 4. Comparison of the most diffused hepatic model systems. Primary hepatocytes cultured in monolayer represent the most diffused model. * HTS: suitable for high-throughput screening.

Since differentiated hepatocytes are anchorage-dependent, immotile and non-proliferating cells, the first "liver-like engineered microenvironments" had, as primary objective, to ensure well-defined characteristics of the substrates (bio/artificial matrices), in terms of architecture (nano-fibrillar), porosity (micro- and macro- scale), and biochemical composition. The products that are now available (even commercially), are hydro-gels and 3D surfaces, composed of specific constituents of original extra-cellular matrices (extractive or synthetic origin), and solid supports (micro-carriers or scaffolds, also pre-shaped), made up of porous, bio-compatible, organic or synthetic components. Examples of the sophisticated models that, for various purposes, have been obtained from primary hepatocytes, are those generated by using bio-degradable nano-structured substrates (Kim *et al.*, 1998), heat-sensitive polymers (Ohashi *et al.*, 2007), various 3D matrices (Fiegel *et al.*, 2008; ZQ. Feng *et al.*, 2010; Ghaedi *et al.*, 2012), or synthetic self-assembling hydrogels (Wang *et al.*, 2008).

The development of the micro- and nano-technologies (i.e. micro-/nano-fabrication techniques, micro-electronics and micro-fluidics), has allowed the creation of models where the cellular elements are integrated into controlled microenvironments, within which, in addition to the precise definition of the spatio-temporal signals individual cells are exposed to, it is also possible to perform the continuous multi-parameter monitoring of their biological responses ("lab-on-a-chip" devices). Representative examples of this approach, applied to cultured hepatocytes, and aimed at generating functional models of liver lobules, bile canaliculi and sinusoids, have been given, respectively, by Ho *et al.* (2006), Lee *et al.*

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(2007), and, more recently, by Nakao *et al.*, 2011. Although structurally very complex and interesting, these models of "micro-structured tissue-like environments" have major drawbacks, that limit significantly their application: they lack, in effect, the complexity of original tissue-specific microenvironments, which are typical of the situation *in vivo*; moreover, they do not allow cell survival for time periods higher than several hours or days.

4. Bioreactors and relative microgravity condition

It is well known that the metabolic requirements of complex 3D cell constructs are substantially higher than those needed for the maintenance of traditional cell monolayers (2D culture) kept in liquid media under static conditions.

The first "dynamic" bioreactors were, in effect, designed in order to meet the necessity of increasing the "mass transfer" rate, for facilitating an adequate long-term supply of gases and nutrients (and the removal of metabolic waste) up to the cellular elements placed in the inner parts of complex 3D tissue explants or tissue-like constructs. Taking advantage of the great progress in the development of new technologies, and of the contribution of computational fluid dynamics, a wide array of dynamic bioreactors have been devised (from the simplest stirred- or suspension-based culture systems, to the more complex membrane-based reactors, and their more sophisticated versions, that include load-, continuously perfused-/pulsed-systems, and multi-compartmentalised bioreactors, able to generate highly controlled microenvironments) (Török *et al.*, 2001, Martin & Vermette, 2005; Catapano & Gerlach, 2007; Meuwly *et al.*, 2007, Guzzardi *et al.* 2011). However, despite all of these technological efforts, none of these bioreactors, are, at present, able to provide optimal conditions for the long-term maintenance of large tissue-like masses in culture. The current generation of bioreactors was, in reality, developed for yielding large masses of cells (or cell products) for industrial or clinical applications, and not for supporting the survival or the self-assembly of multiple cell types into complex 3D tissue-like structures (Hutmacher & Singh, 2008). This applies, in particular, to the liver, a highly specialized organ, whose cellular components are extremely sensitive to even minimal environmental changes, already under physiological conditions (Nahmias *et al.*, 2006; Catapano & Gerlach, 2007).

An important aspect, essential for the appropriate choice of the specific device to use for cell/tissue culture methods, is the consideration that, even if hydrodynamic forces effectively increase mass transfer, in dynamic bioreactors for 3D culturing this effect should be achieved by considering (and balancing) the detrimental effect of turbulence and shear stress on cell survival and function. Low-shear environment and optimal mass transfer have been attained only with the introduction of the Rotary Cell Culture System (RCCS™, Synthecon, Inc.) bioreactors. This technology, fruit of N.A.S.A.'s Johnson Space Center technological research and optimised over the last ten years, has been successfully used in ground- as well as in space-based studies on a wide variety of cell types and tissues (a vast literature is available at http://www.synthecon.com). RCCS™ bioreactors provide several advantages, when compared to other available 3D culture systems (Mazzoleni & Steimberg, 2010). A comparison of the main features of various bioreactors (static or dynamic flow condition) commonly used in the foremost culture techniques is presented in Table 4.

Horizontally rotating, transparent clinostats, RCCS™ devices efficiently create a unique, highly controlled microenvironment that, by reproducing some aspects of microgravity

(simulated microgravity) (Klaus, 2001; Ayyaswamy & Mukundakrishnan, 2007), guarantee the most favourable conditions for cell and tissue culturing (Schwarz *et al.*, 1992), and provide potentially powerful tools to reproduce specific 3D tissue morphogenesis (Mazzoleni *et al.*, 2009). Complex tissue-like 3D constructs, different cell types from various origins and various intact tissue explants have been demonstrated, by our group and others, to be kept efficiently in culture by these bioreactors, even for long periods of time (Unsworth & Lelkes, 1998; Hammond & Hammond, 2001; Vunjak-Novakovic *et al.*, 2002; Nickerson *et al.*, 2007; Cosmi *et al.*, 2009; Steimberg *et al.*, 2009, Steimberg *et al.*, 2010; Mazzoleni *et al.*, 2011).
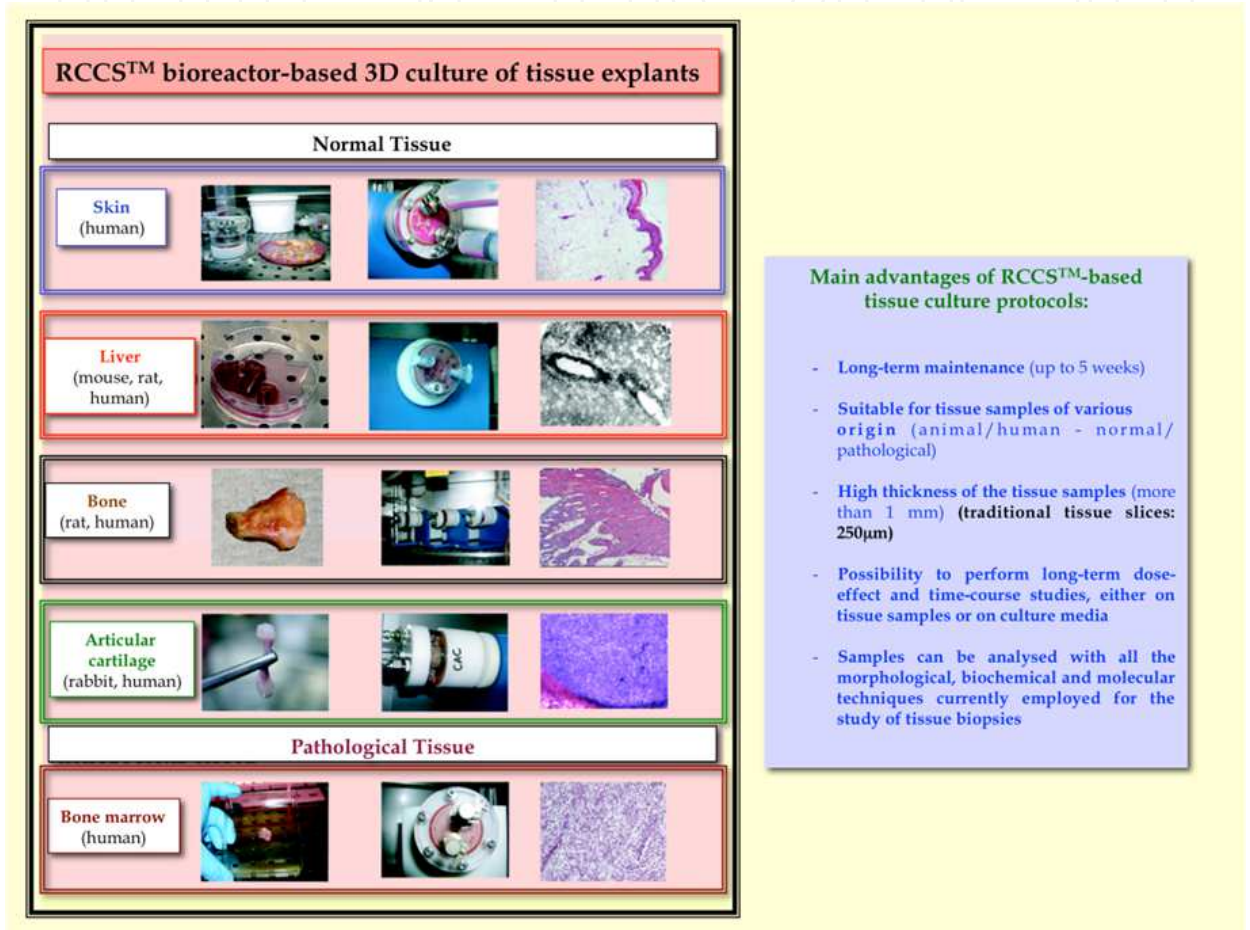
Figure 5 shows selected examples of RCCS™-based tissue culture methods, developed and optimised by our group, and their advantages.

	Shear stress	Mass transfer (gas/nutrient supply; waste removal)	Dimensionality	Adequate for co-culture	Maintenance of liver- specific phenotype
Static 2D culture (monolayer)	No	Adequate	2D	Moderately	Few hours/ days
Static 2D culture (on biosynthetic matrices)	No	Limited	3D (limited)	Moderately	Few days/weeks
Roller bottles	Moderate	Moderate	2D	Poorly	Few days
Spinner flasks	High	High	3D (very limited)	Poorly	Few days/weeks
Microfluidic bioreactors	Moderate/ High	High	2D/ 3D	Moderately	Few days/weeks
Perfused bioreactors	High	High	3D (very limited)	No	Few weeks
Zonation-based devices	High	Optimal	3D	To some extent	Several days
Hollow fiber bioreactors	Low	High	3D (very limited)	To some extent	Some days/ few weeks
RCCS™ bioreactors	Low	Optimal	3D	Yes	Some weeks

Table 4. Static and dynamic bioreactor platforms used in 2D and 3D culture methods.

Even in the case of liver models, it has been demonstrated how these bioreactors allow for the maintenance of isolated human primary liver cells and tissue explants *in vitro*, under conditions that preserve their viability and differentiated functional characteristics, even for long periods (several weeks). Khaoustov *et al.* (1999) have, for example, showed that human hepatocytes, adherent on small biodegradable substrates, were able to survive for 60 days, form specific junctional complexes and structures similar to bile canaliculi, and retain the ability to biosynthesize proteins. The microgravity-based culture conditions generated by the RCCS™ bioreactor have also proved to ensure the long-term survival and preservation of the differentiated metabolic functions in the case of isolated hepatocytes in monotypic 3D

culture (cellular spheroids) (Dabos, 2001), in co-culture with endothelial cells (heterotypic cultures), and in the more complex case of liver tissue homogenates, as well (Yoffe, 1999). Similar observations have also been reported by our group, proving that, under microgravity conditions, primary isolated hepatocytes organize themselves autonomously into multicellular spheroids, with properties similar, for many aspects, to normal liver cells *in vivo* (Mazzoleni *et al.,* 2008). Finally, Wurm *et al.* (2009) have also described an interesting application of the RCCS™ technology for its possible use in the clinical field, as an alternative to conventional models of bioartificial liver (BAL).

Fig. 5. Examples of RCCS™-based tissue culture methods, developed and optimised by our group, and their advantages.

5. Conclusions

The 3D culture methods based on the use of the dynamic RCCS™ bioreactor demonstrates that they can guarantee the best conditions for generating *in vitro* microenvironments suitable for the long-term maintenance of viable liver-derived parenchymal cells and tissue explants. These techniques enable, moreover, to preserve some of the native and typical morpho-functional characteristics of the organ *in vivo*. For these reasons, the RCCS™-based 3D culture methods illustrated in this chapter present, in the current scientific context, the most promising prospect for the development of physiologically significant liver models, which may, in the future, be usefully employed in basic and applied research, in pharmaco-

toxicology, risk assessment, and in clinical fields. Remarkably, the rapid progress in the development of new experimental protocols and analytical methods, together with the possibility of using this innovative microgravity-based culture technology for the development of liver-derived complementary *in vitro* models, based on the use of human isolated cells or intact tissue explants (healthy or pathological), could, indeed, open new perspectives for the study of important (and still unknown) aspects of the patho-physiology (functions and responses) of this complex organ. Once optimised (and validated), these *in vitro* (cell-based) and *ex-vivo* (tissue-based) human liver models should also allow new applications in the field of pharmaco-toxicology and risk assessment, and, in addition, could permit to reduce the need of experimental animals. By mimicking human liver functions and responses, these models could, in effect, be used either for studying mechanisms of toxicity (identification of critical toxicological pathways/targets), or, if adequately optimised, for screening purposes (Blaauboer, 2008).

This is in line with the declared policies of the European Union (EU) and of the United States (US), which solicit more innovative approaches to toxicity testing and the reduction of animal-based studies, as it is well expressed, for example, by EU legislation (7th Amending Directive 2003/15/EC to Council Directive 76/768/EEC on Cosmetics; REACH Regulation on Chemicals and their safe use - EC 1907/2006, Council Regulation n.440/2008 on dangerous substances, and Directive 2010/63/EU on the protection of animals used for scientific purposes) (Lilienblum *et al.*, 2008; Hartung, 2010), and by the 2007 landmark report of the US National Academy of Sciences "Toxicity Testing in the 21st century. A vision and a strategy" (NRC, 2007). The NCR 2007 report emphasizes the need of replacing traditional animal-based studies with innovative testing strategies (physiologically relevant *in vitro* assays and specific *in silico* models), which, taking advantage of new advances in scientific knowledge and new technologies, could improve real exposure measurement and human health risk assessment. The report envisions a shift of the traditional paradigm of toxicology from the measurement of apical endpoints in animal models, to the proper understanding of primary toxic mechanisms ("toxicity pathways") in humans and use of computational modelling techniques ("*in silico* methods"). This vision has generated various research initiatives and several on-going projects, such as the HESI "Risk Assessment in the 21st Century" Project (RISK21) and the US Environmental Protection Agency (EPA)'s ToxCast™ program (Dix *et al.*, 2007; US EPA, 2009), aimed at advancing toxicology strategies in EU and US (Vanhaeke *et al.*, 2009; Stephens *et al.*, 2012). According to NRC 2007 vision, predictive models that need to be developed, should be based on the identification, analysis and modelling of pathways involved in human cellular responses during the transition from physiological to pathological status in response to toxicants (US EPA, 2009). Innovative tissue-specific *in vitro* models (such as the 3D RCSS™-based liver models described in this chapter can be considered), are intended to identify and evaluate key toxicity pathways perturbations (NCR, 2007), in order to create the knowledge base required to develop *in vitro* and *in silico* pathway assay test systems relevant to human risk assessment (Stephens *et al.*, 2012). The specific interest that these 3D liver models present within this new vision in toxicological risk assessment is clearly highlighted by the US EPA Strategic Plan for the Evaluation of the Toxicity of Chemicals (US EPA, 2009), in several parts of the document and, namely, when it refers to the importance of preserving the original 3D tissue-specific heterogeneous microenvironment for improving the predictive potential of *in vitro* systems ("*Some toxicities are manifest only when multiple cell types and specific cell-cell interactions are*

present. Other toxicities may be dependent upon tissue geometry and 3D architecture. Examples include signalling between hepatocytes and Kupffer cells, or the many forms of signalling between epithelial and mesenchymal cells.") (US EPA, 2009).

Ultimately, microgravity-based 3D culture methods could lead to the development of new devices, suitable to support, from the clinical point of view, the liver's essential functions (e.g. new-concept "bio-artificial" livers), or lead to the design of innovative protocols for the autologous transplantation of normal or engineered hepatocytes, in order to counteract liver disorders (e.g. those caused by specific enzymatic defects).

6. Acknowledgments

7. References

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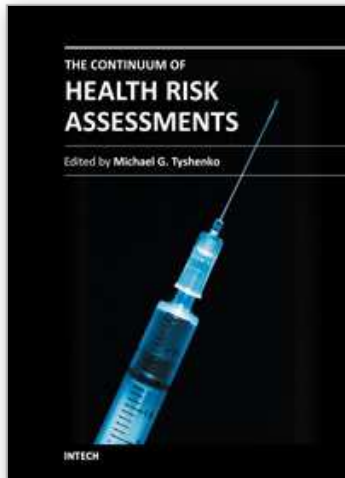
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