Chapter 1

INTRODUCTION
1.1 LIVER DISEASE AND LIVER TRANSPLANTATION

Over 21 million people in the world are estimated to live with chronic liver disease, and about 800,000 expire annually. In the European Union and United States of America over 81,000 and 26,000 people died of chronic liver disease in 2006, respectively\textsuperscript{1,2}. Regardless of the frequently slow progression of the disease, liver transplantation remains the definitive treatment for end-stage liver failure, as it is also the only treatment for severe acute liver failure and for some forms of inborn errors of metabolism.

Although liver transplant has become a procedure with a relatively good 5-year survival, organ donation has not kept up with demand. This fact explains an increasing number of patients on the liver transplant waiting list and waiting longer for a donor organ, which leads to increased morbidity and mortality. In 2007 in the US, there were 5,940 patients transplanted. Although by the end of the year there were still 17,157 people in the waiting list for liver transplant (Fig.1.1). Similar circumstances can be observed in the European Union where 4,779 patients were transplanted by the end of December 2006 and an estimated 20,000 people were still waiting for a donor\textsuperscript{3,4}.

![Figure 1.1](image.png)

**Figure 1.1** Number of patients in Waiting List vs Transplanted until 2007 in the USA. Since the early 90s the gap between available livers for transplantation and patients in the waiting list has widened considerably.
The etiologies of end-stage chronic liver disease that lead to transplantation are numerous and ~80% of people in the liver transplant waiting list have as primary diagnosis a cirrhotic liver (Table 1.1). Fortunately some of the causes of the disease are nowadays preventable. A good example is the successful vaccination programs in many countries in the world against Hepatitis B virus that have considerably reduced the incidence of chronic carriers and viral induced cirrhosis. Regrettably, close to 20% of the livers transplanted in the USA and 30% in Europe have a preventable underlying cause, alcoholic liver disease. Also ~45% of deaths due to liver cirrhosis in the USA are related with alcohol abuse. Patients with pathologies like hepatic cancer, congenital malformations and metabolic diseases, and acute hepatic necrosis compose the remaining percentage of the list.

Table 1.1 | Liver transplantation waiting list patient characteristics in the USA. By the end of 2005, this is the distribution of patients (in percentage) in the liver transplantation waiting list according with their primary diagnosis.

<table>
<thead>
<tr>
<th>PRIMARY DIAGNOSIS</th>
<th>DISTRIBUTION OF PATIENTS IN THE LIVER TRANSPLANTATION WAITING LIST - 2005 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON-CHOLESTATIC CIRRHOSIS</td>
<td>71.9</td>
</tr>
<tr>
<td>CHOLESTATIC LIVER</td>
<td></td>
</tr>
<tr>
<td>DISEASE/CIRRHOSIS</td>
<td>10.4</td>
</tr>
<tr>
<td>BILIARY ATRESIA</td>
<td>4.2</td>
</tr>
<tr>
<td>ACUTE HEPATIC NECROSIS</td>
<td>1.7</td>
</tr>
<tr>
<td>METABOLIC DISEASES</td>
<td>1.7</td>
</tr>
<tr>
<td>MALIGNANT NEOPLASMS</td>
<td>1.2</td>
</tr>
<tr>
<td>OTHER</td>
<td>8.7</td>
</tr>
<tr>
<td>UNKNOWN</td>
<td>0.2</td>
</tr>
</tbody>
</table>
1.2 LIVER ANATOMY AND PHYSIOLOGY

The severity of end-stage liver disease is directly associated with the central role that the liver plays in metabolic homeostasis. It is the largest gland in the body and serves as primary regulatory site for energy metabolism, taking up and processing ingested nutrients for controlled distribution to extrahepatic tissues. Moreover, it also synthesizes vital proteins, enzymes, and cofactors required for digestion and normal bodily function. Finally, the liver is responsible for the detoxification and elimination of a variety of endogenous and exogenous compounds. Its organization is adapted to these functions. It is located in the circulation between the digestive tract and the spleen, receiving all blood from these abdominal organs via the portal vein. It also receives oxygen-rich blood from the hepatic artery.

The blood percolates through the liver in sinusoids that weave between hepatocytes, the main cell type of the liver. Hepatocytes take up amino acids, carbohydrates, lipids, xenobiotics, and other compounds subjecting them to biotransformation with the primary objective of maintain metabolic homeostasis in the body. As an exocrine organ the liver is responsible for the metabolization and secretion of the most hydrophobic waste and toxic substances in the body via the formation of bile. Local regulation of blood flow, secretion of bile and metabolic biotransformation constitute the major functions of this organ. The liver also has an important immunologic function. The vast blood flow that passes through the liver and the high surface area of the hepatic sinusoids enable the liver to be the major organ for phagocytosis of circulating material, including foreign or senescent macromolecules and microorganisms such as bacteria.

Hence, the liver can be viewed as having the following functions and associated structures:

1. Dual blood supply of portal blood derived from the intestinal (splanchnic) circulation, and arterial blood via the hepatic artery.
2. An enterohepatic circulation of substances, in which selected molecules secreted into the bile by the liver (especially bile salts) can be reabsorbed by the intestine and returned directly to the liver via the splanchnic circulation for resecretion into bile.

3. A distinctive architectural arrangement of the intrahepatic vasculature into sinusoids interposed between hepatocytes to facilitate maximal interchange of material between blood and hepatocytes.

4. A unique lining of the sinusoids with specialized endothelial cells containing holes or fenestrae (open onto the perivascular space of Disse), that places fluid in direct contact with hepatocytes.

5. Highly polarized biochemical and functional specialization of hepatocellular plasma membrane domains and subcellular organelles that allow for compartmentalization of specific functions within the hepatocyte and specific interaction with blood, bile, neighboring cells, and the extracellular matrix.

6. Combined expression of exocrine secretory (into both blood and bile) and metabolic functions within hepatocytes.

7. Separation of the biliary compartment from blood to ensure an exocrine pathway directed toward the intestine.

Anatomically, the conventional division of the liver into left, right, caudate and quadrate lobes is a typographic classification that does not correspond to the functional lobes or segments of the liver. The liver is functionally organized in 8 segments, constituting larger units called sectors (Fig. 1.2). The later are demarcated on the external surface by the hepatic scissurae and the umbilical fissure, through which run the hepatic veins. Each segment has its own independent vascular and biliary supply and venous drainage, making their precise surgical dissection determinant in split-donor and living-donor transplantation and in hepatic tumor resection⁹.
1.3 LIVER FUNCTIONAL UNIT – THE ACINUS

There are several definitions for the smallest functional unit (at the microscopic level) of the liver, although the acinus of Rappaport\(^\text{10}\) is probably the most usually described. It is hexagonal in shape and with six sets of portal triads (hepatic vein and artery, and bile duct) demarcating the corners of the hexagon with the central vein at the very center of the acinus (Fig. 1.3). The portal and arterial bloods mix flowing across the plates of liver cells extended between the portal triads and the central vein and leave the liver via the central vein, which is connected then to the vena cava. The blood flow across the liver is in average 1,500 ml/min, constituting 25 % of cardiac output. This is subdivided, with 75 % being supplied by the portal vein, and the remaining 25 % supplied by the hepatic artery. Thus, there is very low shear stress in the blood flow across the cells.

By convention, the liver acinus is demarcated into three zones: zone 1 is periportal; zone 2 is midacinar; and zone 3 is pericentral. The properties of the cells vary, in gradient fashion, along those zones. The smallest cells are diploid and located in zone 1 while the largest cells are polyploidy and located in zone 3. The cell division potential also differs along the acinus being
maximal periportally and negligible pericentrally. Specific genes are expressed in characteristic zones and can be interpreted as in direct correlation of the differentiation stage of the hepatocyte accordingly with the maturational lineage system\textsuperscript{11}. The cells in zone 1 are usually more immature than the cells in zone 3, expressing different enzymatic content and subcellular structures which provide them different metabolic capacities. A good example of this is the prevalence of highly specialized drug metabolizing cells in the pericentral areas of the acinar units\textsuperscript{12, 13}.

\textbf{Figure 1.3} Acinar microstructure. Schematic representation of the acinar structure described by Rappaport\textsuperscript{10}. The arrows indicate the direction of the flow. Portal (blue) and arterial (red) blood mix in the sinusoids and flow in direction of the central vein. The bile (green) flows in the opposite direction, towards the portal triad bile duct.

From:http://www.niaaa.nih.gov/Resources/DatabaseResources/QuickFacts/Liver
1.4 **HEPATOCYTES**

Hepatocytes constitute the major cell type in the liver. They represent about 80% of the total number of cells present, estimated in the human liver to be $100 \times 10^9$ cells\(^7\). They are the workhorse of the liver, executing most of the functions that distinguish the organ and possess also a pivotal role, with endothelial cells, in most regenerative medicine strategies. They arrange in plates or laminas with a single cell layer and have three functionally specialized domains with different receptors, enzymes and molecular transport systems; basolateral and canalicular domains contain site-specific transporters for inorganic and organic ions and major endocytic machinery for uptake of macromolecules; lateral domain contains tight junctions that separate the bile canaliculus from the sinusoids\(^14\).

The hepatocyte is responsible for the secretion of several essential serum proteins like albumin, fibrinogen, and clotting factors, just to mention a few. It is also the major site for the synthesis of lipoproteins, ceruloplasmin, transferrin, complement and glycoproteins. Synthesis of proteins is undertaken by the rough endoplasmic reticulum (RER), and both the rough and smooth endoplasmic reticulum (SER) are involved in secretion of the proteins formed. The endoplasmic reticulum (ER) is involved in conjugation of proteins to lipid and carbohydrate moieties synthesized by, or modified within, the hepatocytes\(^8\).

Besides its secretory functions, the liver generates fatty acids from carbohydrates and synthesizes triglycerides from fatty acids and glycerol. Hepatocytes also synthesize apoproteins with which they then assemble and export lipoproteins (VLDL, HDL)\(^8\).

The liver receives many lipids from the systemic circulation and metabolizes chylomicron remnants. It also synthesizes cholesterol from acetate and then further synthesizes bile salts. The liver is the sole site of formation of bile salts\(^15\).

Most importantly, hepatocytes have the ability to metabolize, detoxify, and inactivate xenobiotics such as drugs and toxins, and endogenous compounds such as steroids. These are some of the functions long sought by the pharmaceutical industry, essential in drug discovery platforms\(^7, 8, 13\).
Other cell types can be found in the liver comprising the remaining 20% of cells. These are endothelial cells, Kupffer cells, stellate cells, and lymphocytes, the population of the hepatic sinusoids and biliary epithelial cells in the bile ducts. For their importance in any tissue engineering approach of vascularized liver tissue and the belief that a transplanted liver construct will be colonized by host hematopoietic cells, endothelial cells will be described in more detail.

1.5 ENDOTHELIAL CELLS

The endothelial cells constitute the wall of the liver sinusoids and account for ~15% of the total number of liver cells. In contrast to the endothelial cells found in other organs, hepatic sinusoidal endothelial cells don’t lie on a basement membrane, but on a discontinuous extracellular matrix derived from hepatocytes, perisinusoidal stellate cells, and the SEC\textsuperscript{16}. The SECs are separated from hepatocytes by the space of Disse, creating a narrow area of extravascular fluid compartment into which hepatocytes project microvilli\textsuperscript{14}.

One of their key features is the numerous pores (0.1um), or fenestrae arranged in clusters, which are known as sieve plates. They allow free exchange of fluids and macromolecular solutes (especially proteins) between sinusoids and the space of Disse, while hindering the passage of particles larger than chylomicron remnants or VLDL. Closure of these pores occurs in some pathological states such as cirrhosis and explains the significantly impairment of hepatic metabolism of large molecular species\textsuperscript{17}.

SECs also possess numerous surface receptors, important for the endocytic uptake of glycoproteins, connective tissue macromolecules, and lipoproteins and, also with function as scavenger circulation, denatured proteins (albumin, ceruloplasmin, iron-transferrin, hyaluronic acid and heparin, among others)\textsuperscript{18}.

Finally, these cells release a variety of cytokines and mediators (e.g. IL-1, IL-6, NO, interferon) that modulate various hepatocyte functions\textsuperscript{19}.

In any tissue engineering strategy, they are also essential due to their primary role avoiding the activation of the intrinsic pathway (contact activation – vessel ECM exposure) of the coagulation
cascade. Hence, any successful approach of vascularized organ engineering has to include endothelial cells.

1.6 LIVER REGENERATION

The ability of the liver to regenerate is remarkable. Epithelial cells in mammalian liver have normally a very slow turn over (1-2 divisions per year), but following surgical resection or toxic injury, the liver regenerates more quickly through compensatory growth of the remained tissue\textsuperscript{20}. Hepatocytes, which are normally quiescent, undergo 1-3 rounds of division followed by division of nonparenchymal cells: biliary epithelial, sinusoidal and stellate cells. The regenerative response is proportional to the mass of the tissue removed. In rodents, after a 2/3 partial hepatectomy, the liver regenerates to its initial size within 1-2 weeks\textsuperscript{21, 22}. Larger animals and humans also undergo liver regeneration although the kinetics is somewhat slower and depend on the extent of the resection and on the presence of coexisting liver disease\textsuperscript{23}.

With such a robust regenerative capacity, it is rather surprising the vast number of patients in the liver transplant waiting list and afflicted by liver disease in general. However, regenerative function unfortunately becomes compromised in many cases of liver malfunction resulting in the need for liver transplant or intensive therapeutic intervention. This impaired regeneration highlights the importance of studying in detail the processes involved in liver regeneration.

There are two distinct forms of liver regeneration: liver regeneration following toxic injuries and liver regeneration after partial hepatectomy. The first type of liver regeneration occurs after a toxic insult to the liver involving the selective loss of mature parenchymal cells in zones 2 and 3. This “cellular vacuum” induces a hyperplasic response from the cells in zone 1. The cells in zone 1 include hepatic progenitors and diploid adult cells that undergo differentiation to the mature parenchymal cells found in pericentral zone\textsuperscript{24}. This phenomenon is the classic “oval cell” response in which small cells with an oval shaped nucleus are induced to proliferate following toxic injury to the liver. This in vivo phenomenon is paralleled by the findings that stem cells in
culture are inhibited by soluble signals released from mature hepatocytes, the “feedback loop”. This “feedback loop” explains why separation of diploid populations away from polyploid ones is required to observe clonal growth of diploid cells in culture and why significant expansion of transplanted liver cells occurs only in hosts in which a “cellular vacuum” exists in the pericentral zone.

The other type of liver regeneration has long been considered to be mediated only by mature liver cells$^{22,25}$. However, it has now been shown to engage the stem cell compartment$^{26}$. In the first 24h after partial hepatectomy, there is a wave of DNA replication across the liver plates, but with limited cytokinesis, resulting in elevated polyploidy and a sharp decline in the diploid subpopulations. The ploidy profile of the parenchymal cells is restored slowly and gradually over several weeks by contributions from the stem cells to the parenchymal cell population$^{27}$.

Recognizing the substantial differences that exist between these two fundamental regenerative processes is crucial in any cell transplantation approach. The existence of the “feedback loop” hinders the transplantation of stem/progenitor cells into livers with still a substantial number of mature cells. Patients suffering from acute liver failure would profit the most with the transplantation of HpSCs or hepatoblast due to the cellular vacuum observed in these patients, allowing these cells to broadly expand.

By contrast, transplantation of these progenitor cells into patients with inborn errors of metabolism should undergo some limited proliferation in parallel with the growth of the patient’s liver cells, but there will be no selection of the transplanted cells over the host cells. Thus, these patients will have intact feedback loop signals and should require much higher doses of cells than patients with acute liver failure.

Transplantation of these progenitor cells to patients submitted to partial liver resection (cancer, split-donor transplant), should result in some expansion of the transplanted cells, with intermediate growth potential between the found in patients with inborn errors of metabolism and that in patients with liver failure.
Finally, the most difficult patient population is likely the patients with liver cirrhosis, who have an aberrant liver microarchitecture containing excessive scar tissue. Engraftment of transplanted cells might be inhibited, and those cells that do engraft will be in a microenvironment that could inhibit their growth and cause them to terminally differentiate. Therefore, strategies for transplantation of stem/progenitor cell populations will be different and depend on the underlying pathological origin in the patient\textsuperscript{28}.

Adult hepatocyte transplantation is another alternative cell therapy in patients with either massive liver failure or inborn errors of metabolism. They have been used with relative success to treat these clinical conditions\textsuperscript{29}. However, several adverse events like portal hypertension, portal vein thrombosis and pulmonary embolism remain problematic, when larger cell numbers are transplanted. Additionally, the scarce availability of human hepatocytes (from organs rejected for transplantation, with reduced viability and functionality) and the fading of their therapeutic action after several months regard them has an unsafe therapy\textsuperscript{30, 31}.

The current therapeutic limitations in cell therapies prompted a more active research in alternative methods such as extracorporeal bioartificial liver devices or liver tissue engineering.

1.7 BIOARTIFICIAL LIVER DEVICES

Bioartificial livers are emerging as a potential therapeutic approach for acute or chronic liver failure or inborn metabolic disorders\textsuperscript{28, 32}. They are projected to be used transiently to rescue patients from acute organ failure with the hope that the patient’s organ can recover from an acute crisis such as a drug overdose or bridge the patient to an organ transplant. Several clinical trials are today ongoing for these types of bioartificial liver devices\textsuperscript{33}.

Most of the bioartificial liver devices being developed are of hollow fiber design. They work similarly to a dialysis machine and allow the blood plasma to flow in the hollow fibers which are coated with viable hepatocytes or loaded with them. Depending on the hepatocyte seeding
configuration, the plasma flows in a distinct compartment of the hepatocytes and never contacts them or flow through them.

Although, the same limitations found on the procurement of livers, for hepatocyte transplantation, are also found here. The limited proliferative capacity of mature hepatocytes makes it difficult to provide abundant cellular mass needed for the patient\textsuperscript{32, 34}. Animal cell sources have also been obtained and used, but the risk of disease transmission rendered them unusable by most regulatory agencies.

1.8 LIVER TISSUE ENGINEERING

Tissue engineering is one of the most promising fields in regenerative medicine. As described in 1993 by Robert Langer and Joseph Vacanti it is the conjugation of biomaterials (synthetic or naturally derived) with cells, in order to generate tissue constructs that can be implanted into patients to substitute a lost function, maintain or gain new functions\textsuperscript{35}. The current paradigm is suitable for the engineering of thin constructs like the bladder, skin or blood vessels. Although, in the specific case of the liver, the 3D architecture and dense cellular mass requires novel tissue engineering approaches and the development of vascularized biomaterials, in order to support thick tissue masses and be readily transplantable. Additionally to the vascular support for large tissue masses, hepatocyte function maintenance represents the ultimate aim in any organ engineering or regenerative medicine strategy for liver disease.

Hepatocytes are known to be attachment-dependent cells and lose rather quickly their specific functions without optimal media- and ECM- composition and cell-cell contacts. Also, function and differentiation of liver cells are influenced by the 3D organ architecture\textsuperscript{36}.

In the last two decades innumerous strategies for the culture of adult hepatocytes in combination with several types of 3D, highly porous polymeric matrices have been attempted\textsuperscript{37-43}. However, in the absence of vasculature, restriction in cell growth and function is common due to the limitations in nutrient and oxygen diffusion. Some of these problems are now partially overcome.
with the development of bioreactors that provide continuous perfusion of culture media and gases allowing a 3D culture configuration and hepatocyte function maintenance\textsuperscript{34,42,44}.

The tissue engineering concept has several advantages over the injection of cell suspensions into solid organs. The matrices provide sufficient volume for the transplantation of an adequate cell mass up to whole-organ equivalents\textsuperscript{45}. Transplantation efficiency could readily be improved by optimizing the microarchitecture and composition of the matrices as well as by attaching growth factors and extracellular matrix molecules to the polymeric scaffold, helping to recreate the hepatic microenvironment\textsuperscript{46}. The use of naturally derived matrices has also proved to be very helpful in hepatocyte culture\textsuperscript{36,39}. These matrices, besides preserving some of the microarchitecture features of the tissues that they are derived from, also retain bioactive signals (e.g., cell-adhesion peptides and growth factors) required for the retention of tissue-specific gene expression\textsuperscript{47, 48}. Additionally, cell transplantation into polymeric matrices is, in contrast to cell injection into tissues and organs, a reversible procedure since the cell-matrix-constructs may be removed if necessary.

Finally, heterotopic hepatocyte transplantation in matrices has already been demonstrated in long term studies\textsuperscript{49, 50}. Nonetheless, initial engraftment rates are suboptimal. One of the reasons for this is the absolute requirement of the transplanted hepatocytes for hepatotrophic factors that the liver constantly receives through its portal circulation\textsuperscript{51}. Thus, the development of a tissue engineered liver construct capable of being orthotopically transplanted is essential.
1.9 OBJECTIVES

Considering the current limitations in the treatment of liver disease and the hurdles found in some of the actual therapeutic approaches, new strategies that can provide improvement in patient morbidity and mortality are required. Cellular therapies and liver tissue engineering represent some of these new strategies, but due to their own technological limitations, they represent a hardly reliable therapeutic alternative.

Some of the limitations have been identified long ago, although definitive solutions remain elusive. Proper tissue construct vascularization is one of them. The complexity in overcoming the oxygen and nutrient diffusion limits in non-vascularized scaffolds hinders the generation of real liver 3D tissue with clinically relevant tissue mass.

The development of vascularized biomaterials represents then a new level of opportunity for the tissue engineering of substantial masses of liver tissue.

The main goal of this doctoral dissertation was to investigate the feasibility of liver tissue engineering using a novel acellular liver derived bioscaffold that preserves its native vascular network.

Evaluation of alternative hepatic cell sources was also pursued by investigating specifically the use of hFL progenitor cells and hAFS cells in liver cell therapy and liver tissue engineering.
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2. CDC. Centers for Disease Control and Prevention Database. CDC. 2007. 2008. Ref Type: Electronic Citation

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6. NIAAA. Age-specific and age-adjusted death rates* for cirrhosis with and without mention of alcohol, United States, 1970–2004. NIAAA.org. 8 A.D. Ref Type: Electronic Citation


