Liver support strategies: cutting-edge technologies

Benjamin Struecker, Nathanael Raschzok and Igor M. Sauer

Abstract | The treatment of end-stage liver disease and acute liver failure remains a clinically relevant issue. Although orthotopic liver transplantation is a well-established procedure, whole-organ transplantation is invasive and increasingly limited by the unavailability of suitable donor organs. Artificial and bioartificial liver support systems have been developed to provide an alternative to whole organ transplantation, but despite three decades of scientific efforts, the results are still not convincing with respect to clinical outcome. In this Review, conceptual limitations of clinically available liver support therapy systems are discussed. Furthermore, alternative concepts, such as hepatocyte transplantation, and cutting-edge developments in the field of liver support strategies, including the repopulation of decellularized organs and the biofabrication of entirely new organs by printing techniques or induced organogenesis are analysed with respect to clinical relevance. Whereas hepatocyte transplantation shows promising clinical results, at least for the temporary treatment of inborn metabolic diseases, so far data regarding implantation of engineered hepatic tissue have only emerged from preclinical experiments. However, the evolving techniques presented here raise hope for bioengineered liver support therapies in the future.

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Introduction

Orthotopic liver transplantation is a well-established procedure for the treatment of end-stage liver disease and acute liver failure. However, whole organ transplantation is invasive, expensive and increasingly limited by the unavailability of suitable donor organs.[1–3] Various alternatives to cadaveric liver donation are being extensively investigated. These alternatives include concepts of artificial and bioartificial liver support. However, despite more than three decades of intense research, the clinical results of these devices still seem to be limited compared with whole organ transplantation. Thus, other concepts such as hepatocyte transplantation and tissue-engineering approaches (for example, recellularization of decellularized organs, organ printing and directed organogenesis) are constantly evolving. With regard to concept-specific limitations, clinical and experimental liver support strategies are affected by two main issues: first, adequate vascularization of the cells and their integration into the host circulation; and second, a source of reliable, safe, highly metabolically active and easily expandable human cells.

This Review focuses on the evolution of artificial, bioartificial and biologic liver support concepts and their current developments. Conceptual limitations are discussed to explain the moderate clinical impact of these concepts compared with whole liver transplantation. Furthermore, the evolving field of hepatocyte transplantation as a less invasive alternative to transplantation is reviewed. Finally, a detailed overview of cutting-edge hepatic tissue engineering is featured. Challenges and opportunities of the different approaches are analysed with respect to clinical relevance, as well as basic science concerns.

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Liver support systems

Artificial and bioartificial liver support systems are extracorporeal devices that are intended to supplement standard intensive care in patients with liver failure by supporting the regeneration of the patient's liver, that is, bridging to regeneration, or by supporting the patient until a suitable organ for orthotopic liver transplantation is available, that is, bridging to transplantation. These devices usually perform the three main functions of the liver: detoxification, regulation and synthesis.

Artificial liver support systems

The hypothesis that the hepatocellular dysfunction present in the clinical syndrome of liver failure is primarily caused by the accumulation of toxins not cleared by the failing liver is addressed by filtration and adsorption devices, so-called artificial liver support systems. In addition to the removal of water-soluble substances, these systems enable the clearance of lipophilic, albumin-bound substances, such as bilirubin, bile acids, metabolites of aromatic amino acids, medium-chain fatty acids and cytokines.

Among artificial liver support systems, two devices have been evaluated quite extensively in the clinic: the Molecular Adsorbent Recirculating System (MARS®; Gambro,

Key points

- Despite almost three decades of research on artificial and bioartificial liver support, appropriate, randomized, controlled, and adequately powered studies are rare
- Compared with the effect of a suitable orthotopic liver graft on a patient's clinical course, the clinical effects of extracorporeal liver support systems seem to be very limited
- The main issues for all liver support therapy concepts are the need for tissue vascularization and integration into the host circulation, and a lack of reliable sources of safe and metabolically active cells
- Strategies to overcome the conceptual limitations of (bio)artificial liver support devices include hepatocyte transplantation and various tissue engineering approaches (for example repopulation of decellularized organs, organ printing and induced organogenesis)
- Hepatocyte transplantation seems to be at least a temporary alternative treatment strategy in certain metabolic liver disorders, and might have a role in the treatment of acute liver failure and chronic liver disease
- Translation of regenerative medicine into the clinical routine, especially transplantation of recellularized liver grafts, seems possible but further intense research is needed

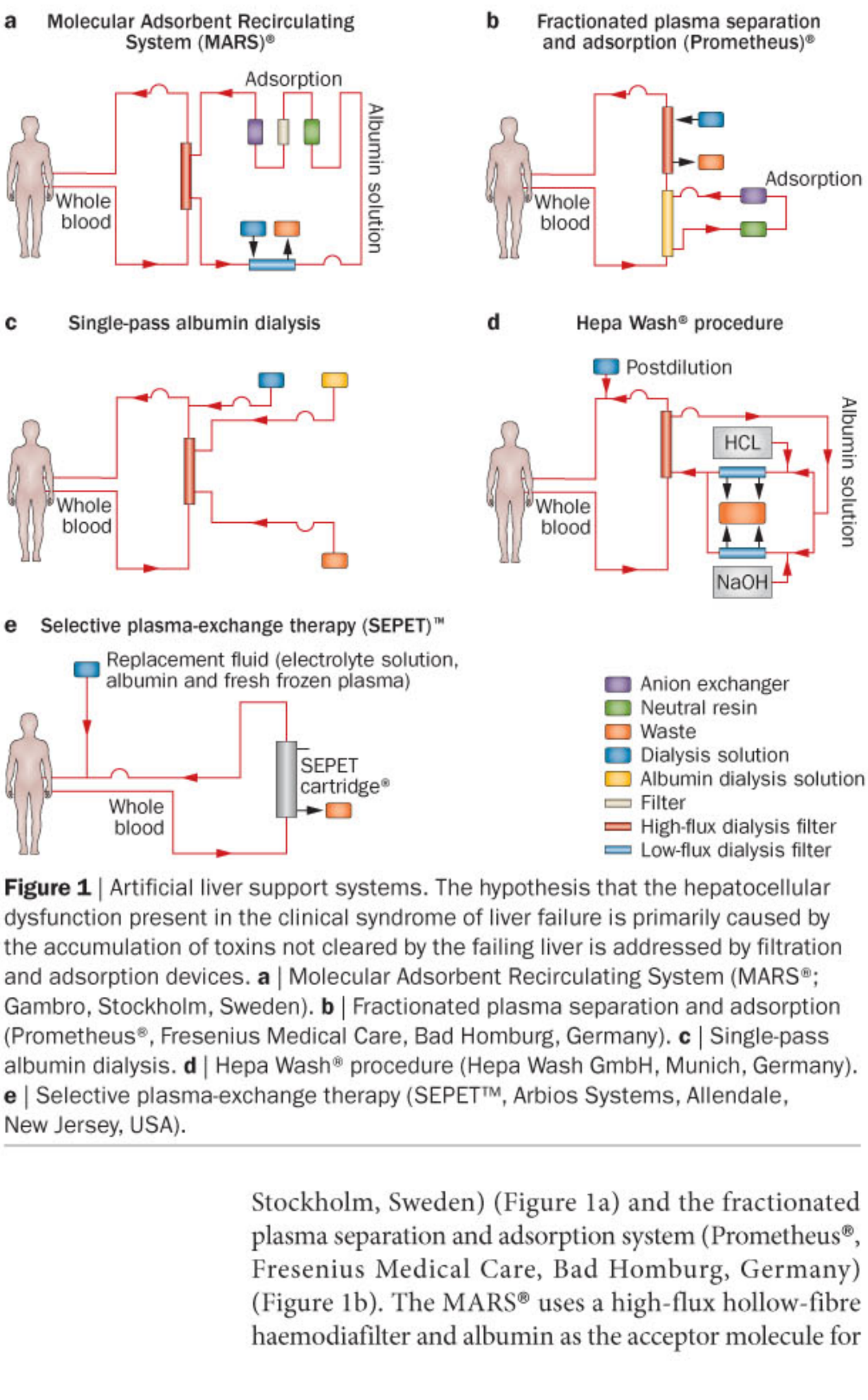

Figure 1 | Artificial liver support systems. The hypothesis that the hepatocellular dysfunction present in the clinical syndrome of liver failure is primarily caused by the accumulation of toxins not cleared by the failing liver is addressed by filtration and adsorption devices. **a** | Molecular Adsorbent Recirculating System (MARS®; Gambro, Stockholm, Sweden). **b** | Fractionated plasma separation and adsorption (Prometheus®, Fresenius Medical Care, Bad Homburg, Germany). **c** | Single-pass albumin dialysis. **d** | Hepa Wash® procedure (Hepa Wash GmbH, Munich, Germany). **e** | Selective plasma-exchange therapy (SEPET™, Arbios Systems, Allendale, New Jersey, USA).

Stockholm, Sweden) (Figure 1a) and the fractionated plasma separation and adsorption system (Prometheus®, Fresenius Medical Care, Bad Homburg, Germany) (Figure 1b). The MARS® uses a high-flux hollow-fibre haemodiafilter and albumin as the acceptor molecule for albumin-bound toxins within the extracorporeal circuit. The recirculating albumin solution is partly regenerated by passing through an anion exchanger and a charcoal adsorber.[4] The fractionated plasma separation and adsorption system is based on an albumin-permeable polysulfone membrane, which enables the patient's albumin fraction to pass into a secondary circuit in which the direct purification from albumin-bound toxins by different adsorbers (that is, anion exchanger and neutral resin) takes place. In addition, conventional, high-flux dialysis is performed within the primary (blood) circuit.[5]

Single-pass albumin dialysis is a simple variation of albumin dialysis using standard renal replacement therapy machines (Figure 1c). Similar to the MARS®, the concept is based on a high-flux hollow-fibre haemodiafilter and an albumin solution in a counter-directional flow. However, in this variation, the albumin solution is simply discarded after being passed through the filter.[6] The Hepa Wash® procedure (Hepa Wash GmbH, Munich, Germany) is a further variation of albumin dialysis (Figure 1d). Instead of regenerating the albumin with adsorbers or discarding it, this procedure uses changes in pH and temperature that enable the albumin to be regenerated.[7] Selective plasma-exchange therapy (SEPET™, Arbios Systems, Allendale, NJ, USA) combines aspects of fractionated plasma separation and adsorption and single-pass albumin dialysis (Figure 1e); the fractionated plasma passes through an albumin-permeable size-selective membrane. Substances of molecular weight <100 kDa are removed, whereas larger molecules—such as immunoglobulins, complement system proteins and most blood-clotting factors—are retained in the blood circulation. The albumin fraction containing the patient's toxins is discarded and replaced by electrolytes, albumin and fresh-frozen plasma.[8] The BioLogic-DT (later Liver Dialysis System™ [HemoCleanse, Lafayette, IN, USA]), which is based on a cellulosic plate dialyzer with a suspension of powdered charcoal and cation exchangers as dialysates, is no longer marketed.[9]

Bioartificial liver support systems

Bioartifical liver support devices aim to combine detoxification with the synthetic and regulatory functions of hepatocytes. To address the complex tasks of regulation and synthesis, the main components of bioartificial liver support systems are extracorporeal bioreactors in which whole livers or liver cells are cultured in a 3D manner within a network of hollow fibres for blood plasma perfusion.

In 1958, Otto *et al.* pioneered extracorporeal liver perfusion (ECLP) in dogs with an Eck's fistula.[10] The first team to use ECLP in a clinical setting was, in contrast to the common belief, not Eiseman,[11] but Sen and co-workers at the University of Bombay, India, in 1964:[12] they treated five patients with ECLP using human livers. Four patients died after <2 days; a 27-year-old patient recovered completely from acute liver failure. Eiseman introduced ECLP using pig livers only 1 month later.[11,13] The clinical application of ECLP is challenging owing to the fact that these livers have to be harvested just in time for the clinical need and have to be connected to

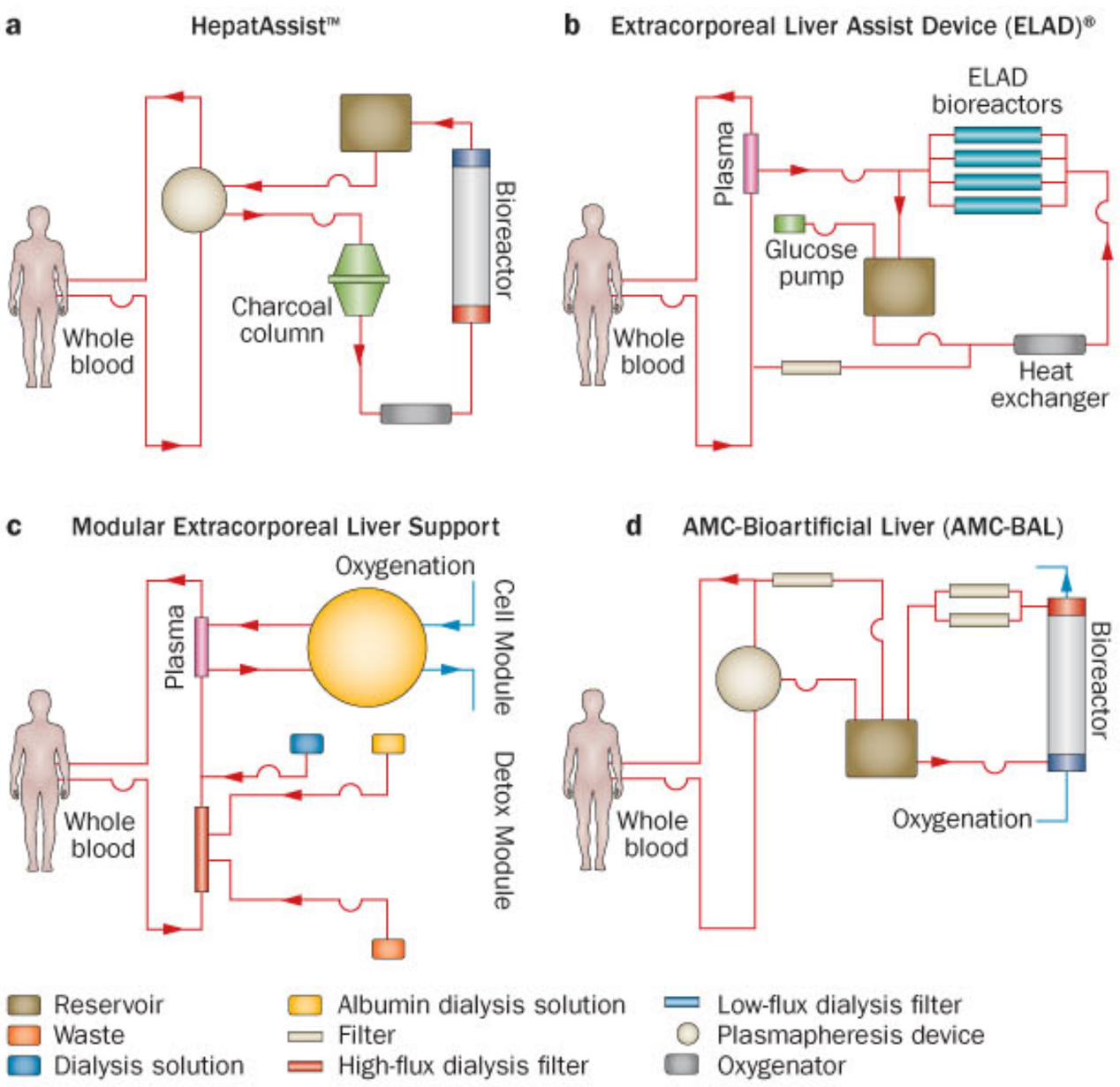

Figure 2 | Bioartifical liver support devices aim to combine detoxification with the synthetic and regulatory functions of hepatocytes. **a** | HepatAssist™ system (Alliqua Inc., Langhorne, PA, USA). **b** | Extracopropeal Liver Assist Device (ELAD®; Vital Therapies Inc., San Diego, CA, USA). **c** | Modular Extracorporeal Liver Support (MELS). **d** | Academic Medical Center Bioartificial Liver (AMC-BAL).

the patient's blood stream usually via a modified heart–lung machine. Bioreactors, which enable the cultivation of isolated liver cells, are easier to handle, can be prepared and characterized in advance and might be integrated into clinically applicable extracorporeal perfusion and dialysis systems. The HepatAssist™ system (Alliqua Inc., Langhorne, PA, USA) is based on cryopreserved porcine hepatocytes within a modified dialysis cartridge (Figure 2a). The patient's plasma ultrafiltrate is led through the cartridges via an activated charcoal adsorber and an oxygenator.[14]

In a similar set-up, the Extracorporeal Liver Assist Device (ELAD®; Vital Therapies Inc., San Diego, CA, USA) developed by Sussman *et al.* uses a hepatoblastoma cell line (Figure 2b).[15] The cells are separated from the patient's plasma by hollow-fibre membranes and an integrated charcoal adsorber, and a membrane oxygenator supports detoxification and maintains the oxygen supply of the cells.[15] A bioreactor introduced by Gerlach *et al.*[16] consists of two bundles of hydrophilic polyethersulfone membranes combined with hydrophobic hollow fibres for oxygenation. The bioreactor was charged with primary pig[17] or human liver cells and integrated into the Modular Extracorporeal Liver Support (MELS) system (Figure 2c).[18] The Academisch Medisch Centrum Bioartificial Liver (AMC-BAL) developed by Chamuleau's group differs from other clinically applied systems in one major aspect; instead of separating the patient's plasma from the liver cells by a membrane, the plasma is in direct contact with the cells, enabling a better mass exchange between cells and the patient's plasma (Figure 2d).[19]

Clinical findings

In general, appropriate, randomized, controlled, and adequately powered studies of artificial and bioartificial liver support systems are rare, and most clinical studies are nonblinded and uncontrolled. Five large prospective randomized controlled trials (RCT) have evaluated MARS®,[20–24] and one is currently recruiting patients with hypoxic hepatitis.[25] Application of MARS® was shown to be safe with only rare adverse events and resulted in improvement of encephalopathy. However, no effect on survival was shown.[20–24] Two studies evaluating MARS® (the RELIEF trial,[24] $n = 189$) and Prometheus® (the HELIOS study,[26] $n = 145$) in patients with acute-on-chronic liver failure have been published. Although both studies showed an acceptable safety profile, no increase in the probability of survival was demonstrated with the artificial liver support systems. Clinical data on single-pass albumin dialysis is limited to case reports and small series.[27–29] Currently, two clinical RCTs evaluating Hepa Wash® are recruiting patients with acute-on-chronic liver failure and hepatic dysfunction.[30,31]

Bioartificial liver support concepts have been evaluated in small studies only,[15,17–19,32,33] with the exception of the HepatAssist™ system. In an RCT of 171 patients with acute liver failure or primary nonfunction after liver transplantation, the 30-day survival rates were 71% versus 62%, respectively, for the HepatAssist™ device compared with standard care ($P = 0.26$). However, survival in the subgroup of patients with fulminant or subfulminant hepatic failure was significantly higher in the HepatAssist group compared with the control group ($P = 0.048$).[14] Currently, two RCTs evaluating ELAD® are recruiting patients with acute alcoholic hepatitis.[34,35]

Three major systematic reviews and meta-analyses on the clinical impact of liver support systems are currently available. A systematic review by Kjaergard *et al.*[36] and a Cochrane review[37] suggested that artificial support systems reduce mortality in acute-on-chronic liver failure compared with standard intensive care. Artificial and bioartificial support systems did not seem to affect mortality in patients with acute liver failure. Stutchfield *et al.*,[38] however, evaluated the role of contemporary extracorporeal liver support devices in patients with acute and acute-on-chronic liver failure through a meta-analysis of randomized controlled studies. They concluded that these systems seemed to improve survival in patients with acute liver failure. There was no evidence that they improved survival in acute-on-chronic liver failure.[38]

Limitations

Compared with the tremendous effect of a suitable orthotopic liver graft on a patient's clinical course, these results from clinical trials seem very limited. With regard to artificial liver support devices, detoxifying the blood of the patient seems to be insufficient—presumably due to the lack of hepatocytes able to address more

sophisticated metabolic pathways. Detoxification seems to be more complex than elimination of albumin-bound toxins; regulation is not limited to regulation of acid–base status and electrolyte levels. Moreover, whether the rather unselective adsorption of substances from the patient's blood might have adverse effects on the regeneration of the failing liver remains unclear—for example, the increase of certain albumin-bound toxins might actually induce liver regeneration, and the charcoal and resin adsorbers might have an effect on the clearance of growth factors and cytokines.

The clinical impact of bioartificial liver support is limited by the lack of an ideal—that is, reliable, safe, highly metabolically active and easily expandable—human cell source. Human hepatoblastoma cells, for example C3A cells used within the ELAD®, are limited by impaired metabolic function and a certain risk of metastatic cell spreading via broken or leaking hollow fibres. Primary porcine hepatocytes (which are used in the AMC-BAL and HepatAssist™ systems) have limited metabolic biocompatibility and have been called into question as an appropriate cell source for clinical application due to their immunogenicity (anti-pig IgG and IgM were seen after clinical application)[17] and risk of xenozoonoses (such as porcine endogenous retrovirus). The inferiority of xenogeneic cell sources is supported by a meta-analysis of data from 198 patients treated with ELCP:[39] pig liver perfusions resulted in lower long-term survival than the use of human livers (20% versus 43%, respectively; $P<0.05$). The use of baboon livers for extracorporeal perfusion revealed superior success compared with pig livers (41% long-term survival). Although the role of hyperacute rejection in acute liver failure with reduced complement levels remains controversial, physiologic disparity between pigs and humans might be the even more decisive determinant of outcome.[39]

Furthermore, bioartificial liver support systems have a major technical limitation: their membranes. Mass exchange is limited due to the artificial membranes separating the patient's blood from plasma and—in most systems—plasma from cells. In addition, owing to resistance of the bundles of hollow fibres, flow rates within the bioreactors are low (100–200 ml/min) compared with those of *in vivo* perfusion (~1,500 ml/min).[40]

On the basis of these issues, alternative strategies have been developed toward a more physiologic method with close contact of hepatocytes to patient's blood, ensuring an optimal mass exchange. These options include cell transplantation, and bioengineering concepts such as the repopulation of decellularized livers, organ printing and induced organogenesis.

Hepatocyte transplantation

Hepatocyte transplantation is the infusion of isolated hepatocytes into the portovenous system.[41] After translocation into the hepatic sinusoids, donor hepatocytes integrate into the liver plates and, under circumstances such as a selective proliferative advantage, repopulate the recipient liver.[42,43] Using this approach, the native liver is left in place and serves as a matrix for the donor cells.[44,45] Hepatocyte implantation is not solely limited to the liver; hepatocytes can also be implanted to 'ectopic' implantation sites such as the spleen or the peritoneal cavity.[46,47] Hepatocyte transplantation can thereby partly overcome the two main problems of artificial and bioartificial liver support. First, the cells are not separated from the patient's blood stream and thus they interact in a more physiological manner compared with a culture in a bioreactor. Second, the shortage of donor organs can be addressed as multiple patients can be treated with hepatocytes isolated from a single donor organ.[48] For example, for inborn metabolic liver diseases, the replacement of 5–10% of the theoretical liver mass with hepatocytes expressing the gene involved can be enough to correct the metabolic disease.[44,49]

Clinical applications

Until now, hepatocyte transplantation as a liver support therapy has only been investigated in small clinical series and case reports.[45] The most promising results have been obtained for the treatment of inborn metabolic liver diseases, which are characterized by a single missing hepatic enzyme or protein[50] and can be corrected with the engraftment of hepatocytes expressing the gene involved. As an example, Crigler–Najjar syndrome type I is characterized by the complete absence of one of the uridine diphosphate glucuronosyltransferase enzymes, resulting in life-threatening unconjugated hyperbilirubinaemia.[51] According to the current literature, eight patients have undergone hepatocyte transplantation for treatment of Crigler–Najjar syndrome type I.[52–57] In all cases, a decrease in serum bilirubin levels of 25–50% could be observed.[52–57] However, the effect decreased over time, and whole liver transplantation was necessary after 4–48 months for all patients due to hepatocyte graft failure.

Urea cycle defects are another example of the successful application of hepatocyte transplantation. This group of diseases is characterized by the absence of one of the enzymes involved in the urea cycle, resulting in hyperammonaemia and subsequent neurological impairments.[48] A total of 10 cases have been reported in which hepatocyte transplantation was performed for the treatment of urea cycle disorders:[48,58–64] six patients with ornithine transcarbamylase deficiency; one patient with argininosuccinate lyase deficiency; one patient with carbamoylphosphate synthase 1 deficiency; and two patients with citrullinaemia. In all cases, ammonia levels could be reduced, but most of the patients had to undergo orthotopic liver transplantation later on. However, two patients died due to severe metabolic decompensation 43 days and 4 months after hepatocyte transplantation. These cases demonstrate that hepatocyte transplantation is feasible, at least for the temporary treatment of distinct metabolic liver disorders. Currently, three clinical studies are listed in the clinicaltrials.gov registry that aim to further investigate the effectiveness of hepatocyte transplantation to compensate for metabolic liver diseases, provide a bridge to liver transplantation or an alternative to whole organ replacement.[65–67]

By contrast, clinical studies of hepatocyte transplantation for acute liver failure have until now shown only inferior results.[50] These poor results might occur because, in acute liver failure, the number of hepatocytes that can be safely transplanted has not been sufficient for hepatic recompensation. Two clinical trials are currently aiming to further investigate whether hepatocyte transplantation can be used as a bridge to orthotopic liver transplantation during acute liver failure.[68,69] In chronic liver disease, in which the liver architecture is damaged and not ideal for engrafting a sufficient amount of cells, the results of the clinical investigation were even worse. Currently, no further clinical studies are investigating this indication.[50]

In the reported clinical cases, hepatocytes have mainly been transplanted directly to the liver via the portal vein. Successful hepatocyte transplantation into the spleen has also been reported, with demonstration of hepatocyte engraftment and hepatization of the spleen.[70–72] Reports on clinical transplantation of hepatocytes to other ectopic implantation sites in humans have not yet been published.

Future directions

Despite promising results from initial clinical investigations, various barriers must be overcome before hepatocyte transplantation can be established as a routine clinical treatment.[73] Similar to bioartificial liver support, the lack of a sufficient source of viable hepatocytes limits the wider application of clinical hepatocyte transplantation.[44] Insufficient engraftment and long-term survival of hepatocytes limit clinical success.[74] Furthermore, immunosuppressive treatment has not yet been optimized for hepatocyte transplantation.[56] Both noninvasive tracking of the transplanted cells and monitoring of their metabolic activity also represent substantial challenges.[63,75]

As protocols for the generation of stem cells or induced pluripotent cells are not yet available for clinical use, a more immediate solution for the shortage of donor organs for hepatocyte isolation could be the use of non-heart-beating donor organs or even cadaveric livers. Hughes *at al.*[76] showed that metabolically active hepatocytes could be isolated from the livers of non-heart-beating donors after a warm ischaemic time of 15 min for use in clinical transplantation. Erker *et al.*[77] demonstrated that hepatocyte isolation is even feasible after 24 h of cold storage at 4 °C, which would further increase the window of time for isolation from cadaveric livers after unanticipated deaths. Another forward-looking approach might be the use of xenotransplantation. Nagata *et al.*,[78] using a Cynomolgus monkey model, were able to show that transplanted xenogenic porcine hepatocytes were metabolically active for >80 days after adhering to an effective immunosuppression regime. As xenogeneic porcine hepatocytes seem to be less immunogenic than whole organs, xenotransplantation could be a potential solution for hepatocyte transplantation.[78,79]

As evidence of long-term hepatocyte engraftment is lacking in most of the past clinical cases, preclinical studies have been performed to develop clinically applicable methods to precondition the recipient liver and to stimulate the proliferation of the donor hepatocytes.[80–82] Preoperative radiation and transient portal vein occlusion are the most promising approaches and are currently being investigated in two clinical studies on hepatocyte transplantation in the treatment of metabolic liver disorders.[66,67] Preoperative radiation is intended to 'make room' for donor hepatocytes engraftment by inhibiting host hepatocyte proliferation and inducing post-mitotic hepatocyte death, and transient portal vein occlusion prior to hepatocyte infusion is used to provide a compensatory mitotic signal to the donor hepatocytes.[73]

Intraperitoneal implantation of encapsulated hepatocytes has been investigated in mice with acute liver failure.[83] This procedure offers more space (within the abdominal cavity) in comparison to the portovenous system. Therefore, this approach might be of interest for further investigation in preclinical and clinical trials as it provides more space for the necessary larger amount of cells that are needed in acute liver failure.[83] In another experimental model, human hepatocytes were implanted within a decellularized liver matrix into the omental tissue of mice for 6 weeks. The authors noted improved hepatocyte survival and function compared with splenic injections.[84]

The cirrhotic liver has previously been considered as not suited for long-term hepatocyte engraftment. However, a study by Yovchev *et al.*[85] raises new hope for patients with cirrhosis. They showed in a rat model that transplanted epithelial stem and progenitor cells could restore injured parenchyma in a liver environment with advanced fibrosis, and could exhibit antifibrotic effects. Another striking approach potentially suitable for treatment of chronic liver disease or acute liver failure is hepatocyte transplantation into lymph nodes.[86,87] Hoppo *et al.*[86] and Komori *et al.*[87] showed that direct injection of hepatocytes into a single mouse lymph node generated enough ectopic liver mass to rescue mice with lethal metabolic liver disease. Ectopic hepatocyte transplantation combined with advanced tissue engineering concepts could open up new ways of hepatocyte transplantation for treatment of patients with end-stage cirrhotic liver disease.

Repopulation of decellularized organs

Many efforts have been undertaken to keep relevant masses of hepatocytes metabolically active outside of their natural environment. As already mentioned, for adequate metabolism, cells require blood vessels within a 1–3 mm range for oxygen and nutrient supply and for the removal of metabolic products.[88,89]

One approach to engineering vascularized, functional tissue is the decellularization and recellularization of solid organs (Figure 3). During decellularization, cells and other immunogenic molecules (for example DNA and alpha-Gal epitopes) are removed from tissue or organs to leave the extracellular matrix. The extracellular matrix is generated by specific protein secretions from resident cells and preserves the complex 3D microanatomy of an organ, including its 'vascular framework' and—in the case of the liver—the framework of the biliary system. Given that extracellular matrix proteins are highly conserved across species, they are unlikely

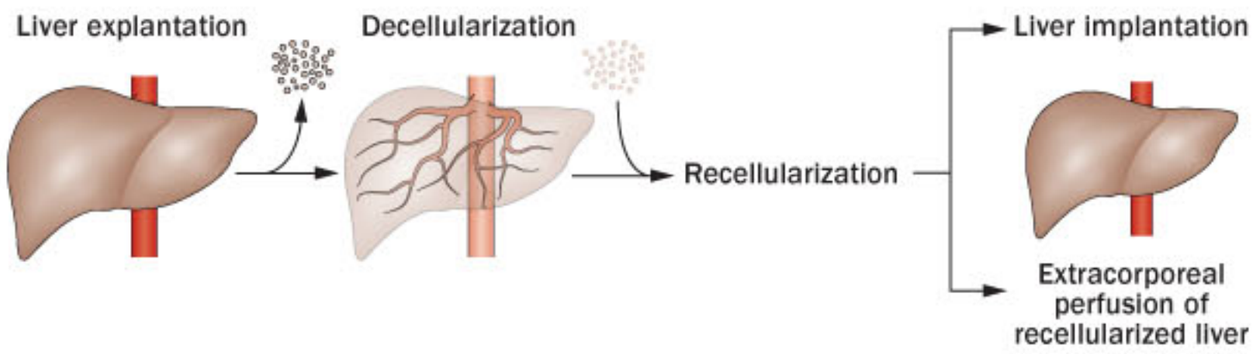

Figure 3 | Concept of recellularization of decellularized livers and consecutive implantation or extracorporeal application. Cells and other antigenic material (for example, alpha-Gal epitopes and DNA) are removed from a liver (porcine for example) to obtain the non-immunogenic extracellular matrix. The extracellular matrix preserves the 3D microanatomy of the liver, including a protein framework of the vascular and biliary system and serves as an ideal biomatrix for cellular repopulation. After recellularization with (stem) cells (for example, from a patient on a waiting list for a liver transplant) the neo-liver is matured in a bioreactor. The functional liver organoid is then implanted into a patient with liver failure or used for extracorporeal perfusion in a liver support device.

to induce immunogenic reactions, even in allogenic or xenogenic host organisms.[90,91] Furthermore, as the extracellular matrix is in a state of 'dynamic reciprocity'[92,93] with local cells, regulating migration and proliferation and able to induce the differentiation of progenitor or stem cells through its specific composition,[94–97] the extracellular matrix represents a biomatrix ideally suited for repopulation with cells.[98] Using this elegant technique, extracellular matrix could be obtained by the decellularization of, for example, porcine livers on a clinically relevant scale. Then, the obtained non-immunogenic biomatrix could be repopulated with the (stem) cells of a patient on a waiting list for liver transplant. Finally, after maturation in a bioreactor, this autologous graft could be implanted without the requirement for lifelong immunosuppressive therapy and its numerous adverse effects.

Badylak and his group[99] were the first to propose a qualitative definition of the term decellularization. However, experimental evidence for the clinical relevance of these criteria is still rare[100–102] and should be further evaluated experimentally. Currently, the decellularization of livers to obtain the extracellular matrix is primarily achieved by perfusion with alkaline detergents (for example, Triton™ X-100 [Dow Chemical Company, Midland, MI, USA], SDS and CHAPS), optionally in combination with enzymatic agents (such as trypsin and nucleases).[99]

Recellularization strategies combine available cell isolation and culture techniques with methods established for extracorporeal organ perfusion: after seeding of cells into the decellularized liver matrix, maturation of the neo-organ in a bioreactor is awaited. So far, the re-seeding of liver extracellular matrix has been initiated either by antegrade (via the portal vein) or retrograde (via the hepatic veins) infusions[88] of different cell types (such as hepatocytes, progenitor cells, stem cells, endothelial cells and stellate cells).[103–105] Moreover, attempts to repopulate the matrix via direct puncture with a cannula and infusion of a cell-spheroid-enriched gel have been published.[103,106] Cell infusion is followed by a phase of engraftment and migration by the cells to their specific niches in the extracellular matrix. The recellularized grafts are then perfused at 37 °C in bioreactors, and the nutrient and oxygen supply of the cells is determined by different compositions of culture media or heparinized blood. The number of cells employed varies depending on the size (and species) of the extracellular matrix; however, this procedure is still limited by insufficient cell resources and high costs for the expansion of progenitor or stem cell lines.

In vivo studies

Data on *in vivo* implantations of engineered hepatic tissue are limited to animal studies, with the first study on the implantation of a recellularized liver graft coming from Uygun *et al.* in 2010.[104] They applied the decellularization technique for hearts published by Ott *et al.*[107] to rat livers and recellularized the median lobe of a rat liver extracellular matrix with primary rat hepatocytes and microvascular endothelial cells.[104] In the first step, recellularized grafts were perfused in a bioreactor for up to 5 days *in vitro* and analysed. These experiments indicated a better viability and function for hepatocytes within the perfused organoids compared with hepatocytes under conventional culture conditions. Then, the engineered grafts were implanted auxiliary in rats with arterial blood flow from the renal artery for up to 8 h. Interestingly, histological staining of the explanted grafts indicated that the hepatocytes had migrated into the extracellular matrix, engrafted around larger vessels and retained their normal morphology and function. Meanwhile, endothelial cells were capable of lining the vasculature.

In the same year, Bao *et al.*[108] recellularized the right median lobe of a decellularized rat liver with rat hepatocyte spheroids and implanted these tissue-engineered livers (TELs) in rats. Interestingly, the authors showed that the mean lifespan of 90% hepatectomized rats improved from 16 h for rats without TELs to 72 h for rats treated with TELs. However, 96 h after implantation, all TEL-treated rats were deceased as well. Possibly the most innovative idea from this work was the application of an inner heparin multilayer technique to coat the TEL with an antithrombotic agent, preventing blood clotting locally inside the graft.[108]

Soto-Gutierrez *et al.*[106] recellularized the extracellular matrix of rat livers with ~$10–50 \times 10^6$ mouse hepatocytes through the direct injection of hepatocytes via puncture or through single-step or multi-step infusion of cells via the portal vein. Recellularized grafts were then perfused for up to 7 days *in vitro* in a bioreactor, and the culture medium and grafts were analysed. In this experiment, recellularization via multi-step infusions through the portal vein showed significantly better results than other techniques regarding hepatocyte engraftment, viability and function, which is consistent with the findings of Uygun *et al.*[104]

Baptista *et al.*[88] then scaled up the decellularization technique for the livers of different species (for example, mice, rats, ferrets, rabbits and pigs) and recellularized a ferret liver scaffold with human fetal liver cells and human umbilical vein endothelial cells (hUVECs). After maturation in a bioreactor for 7 days, the researchers perfused the grafts with heparinized rat blood for 30 min *in vitro* and found fewer aggregated platelets in the scaffolds

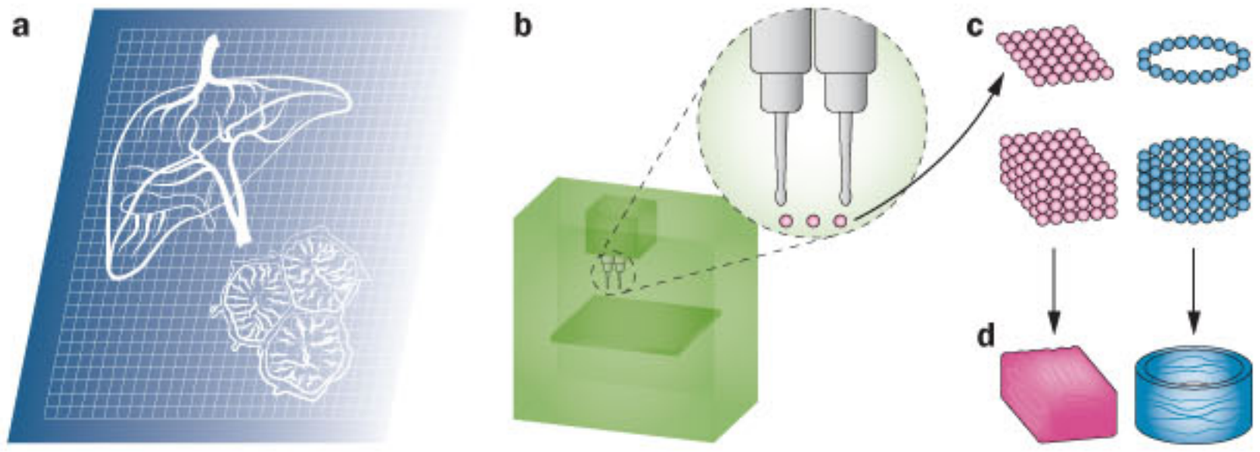

Figure 4 | 3D organ printing. The most recent organ printing techniques are conceptually identical to industrial 3D printing techniques (that is, rapid prototyping) and can be defined as layer-by-layer additive biofabrication using liquid bioink (cell suspensions) or self-assembling cellular building blocks (spheroids). Printing consists of four different steps. **a** | Pre-processing. Generation of a computer-aided design based blue-print. **b** | Processing (printing). Dispensing of cell droplets or spheroids (and hydrogel or other extracellular factors). **c** | Post-processing (maturation). Printed cells form tissue-like blocks or ring-like structures. **d** | During maturation (in a bioreactor) cells self-organize, start cell–cell interactions and secrete extracellular matrix to finally become functional solid tissue (for example, parenchyma) or tissue tubes (such as blood vessels or bile ducts).

re-seeded with endothelial cells than in unseeded scaffolds; this result underlines the importance of endothelial cell coverage of the vascular lumen. Furthermore, they found bile-duct-like structures and hepatocyte cell clusters and determined that the extracellular matrix supported the differentiation of foetal hepatoblasts into biliary and hepatocytic lineages.[88] Barakat *et al.*[105] 'humanized' the posterior segments of decellularized porcine livers by infusions of human fetal hepatocytes with co-cultured human fetal stellate cells and perfused these grafts for up to 13 days *in vitro*. Inside the extracellular matrix, the human fetal hepatocytes differentiated into mature hepatocytes and bipotential progenitor cells, leading the researchers to the idea that the biliary epithelium could have been re-assembled after longer periods of perfusion by transdifferentiation induced by the extracellular matrix.

Future directions

The potential of the decellularization and recellularization methodology has been demonstrated in its first clinical applications in other areas of medicine,[109–114] although extensive basic research is required before tissue engineering concepts can be applied broadly in clinical practice.[115] However, it seems possible that extracellular matrix of xenogeneic origin or from marginal organs unsuitable for transplantation could be repopulated with autologous cells from patients on a transplant waiting list. As such, the organ shortage could be overcome and the various adverse effects of lifelong immunosuppressive therapy would be minimized or even avoided because the engineered grafts would consist mainly of the patient's own cells.

Although in general implantations of recellularized livers in humans seem possible, various issues still have to be resolved. Similar to one of the major obstacles in designing bioreactors for extracorporeal liver support, reconstruction of vascular integrity (that is, re-epithelialization) is the limiting step: contact between the extracellular matrix proteins and blood components serves as a major trigger for the formation of blood clots. Thus, implantations of recellularized grafts will not be possible until the vasculature is completely re-epithelialized or thrombogenesis within the graft is prevented in a different manner without deleterious systemic effects on the host. However, the fact that the vascular extracellular matrix frame is conserved during decellularization is possibly the biggest advantage of this technique in comparison to other tissue engineering approaches.

Preclinical experiments and clinical trials are needed to define relevant quality standards to predict the *in vivo* behaviour of decellularized tissue of different species in humans. Furthermore, animal studies are needed to completely understand the sophisticated interactions of repopulated cells and the extracellular matrix. The expansion and directed differentiation of embryonic, fetal or (autologous) induced pluripotent stem cells[116–118] might be a viable solution to open up a potentially indefinite cell source for recellularization but is technically and economically not feasible until the applied techniques are safe and more efficient.[105,119]

Tissue and organ printing

Techniques

An even more radical approach to enable the precise engineering of complex parenchymal organ constructs, including hierarchically branched vascular trees, is 3D organ printing (Figure 4).[120] The idea of manipulating the fate of living cells by printing biologic material (such as collagen and fibronectin suspensions) and cells was first published by Klebe in 1988.[121] On the basis of this pioneering work, numerous kinds of printing techniques have emerged to print scaffolds, material to influence cells, different cell types or even material that mimics living tissue.[122,123]

The most recent organ printing techniques are conceptually identical to industrial 3D printing procedures (such as rapid prototyping), during which solid objects are constructed on the basis of computer-aided designs by additive manufacture of successive layers in different shapes or voxels (volumetric pixels). Thus, 3D organ printing can be defined as layer-by-layer additive biofabrication using liquid bioink (that is, cell suspensions; equivalent to layer-addition) or self-assembling cellular building blocks (that is, spheroids; equivalent to voxels).[124,125]

3D spheroid printing represents scaffold-free tissue engineering (a 'bottom-up' technique) and enables the precise arrangement of different cell types and other biologic materials (such as extracellular matrix components and growth factors) within organ constructs.[126] Furthermore, by using this technique, cells are cultured more closely to '*in vivo* conditions', compared with traditional cell culture techniques, which enhances intercellular communication and avoids dedifferentiation of sensitive cells such as hepatocytes.[122] The spheroid printing technique is based on tissue fusion, a process driven by tension forces between fluids that is observed broadly throughout embryonic development.[127,128] Tissue fusion means that spheroid blocks of different living cell types that have been placed closely together by a bioprinter melt together to finally represent one entity.[126] In this manner, tissue spheroids comprised of endothelial and

smooth muscle cells can be used to print ring-like constructs, which ultimately fuse to form functional blood vessels.[125] Then, the application of spheroids of different sizes enables the construction of vessels with different diameters, ultimately creating a segmented vascular tree within an engineered parenchymal organ.

Although no reports on printed perfusable hepatic constructs have been published so far, some interesting experimental results demonstrate the potential of 3D printing techniques.[129,130] Robbins *et al.*[131] used the NovoGen MMX Bioprinter™ (Organovo Holdings, Inc., San Diego, CA, USA) to print metabolically active 3D hepatic tissue. They demonstrated increased liver specific function of the tissue for up to 135 h compared with matched 2D cell cultures. Furthermore, compartment-specific organization in a rudimentary microanatomy was shown for hepatocytes, hepatic stellate cells and endothelial cells.

Sun and his group used the solid free-form fabrication technique to print alginate-encapsulated HepG2 cells, growth factors and scaffold materials in an organized 3D architecture.[132] These micro-organs were dynamically micro-perfused to mimic an *in vivo* scenario for drug metabolism studies. As a follow-up, the authors used this system to perform a radioprotection study on liver cells.[133]

Barriers to overcome

However, these hepatic micro-organs are not designed as organ supportive therapy options. Unfortunately, building structures that are analogous to tissue in their composition is not the same as developing fully functional tissue,[122] and bioprinting techniques still have to overcome various implemented issues before translation into the clinic.

First, methods to gain detailed knowledge of an organ's 'micro-anatomy', and software to convert this information into reasonable blueprints are prerequisites to print functional 3D organs; these methods are being constantly improved.[134,135] Furthermore, complex bio-mathematical models to predict the behaviour of biological materials (for example, liquids of different viscosities, heterogeneous cell mixtures and charged molecules) before, during and after printing are needed; a PhD track in applied mathematics has been established at the University of South Carolina, USA, to develop these models.[135–137]

The enhancement of available printing hardware to quickly process living cells is a determining step, as the printing process affects the viability of cells[138] and cells might also have an effect on the printer.[130,139] Today, printing of a tissue block of 1 cm^3 takes up to 27 h, indicating the technical limits of currently available printing techniques.[140] Moreover, maturation processes further limit the scalability of printed tissue, as it can take months until engineered tubules of printed spheroids are functional and perfusable.[135] Thus, sustaining the viability of parenchymal cells during maturation and essentially accelerating maturation are fields of particular interest, especially as most experiments so far have been performed with young animal cells—human primary cells are even more complex to handle *in vitro*.[130]

An elegant hybrid approach to generate perfusable tissue within minutes by combining industrial 3D printing and traditional, scaffold-based cell culture techniques was published in 2012.[141] The authors encapsulated a 3D-printed carbohydrate glass lattice with extracellular matrix and living cells and consecutively dissolved the lattice to obtain a monolithic extracellular matrix with perfusable channels. These channels were 'endothelialized' with hUVECs, resulting in a tissue construct that was perfusable with human blood. To further assess the value of this approach for sensitive cells, they engineered gels containing primary rat hepatocytes. After 8 days of *in vitro* culture, hepatocytes in perfused gels showed better survival and substantially higher albumin secretion and urea synthesis than hepatocytes in unperfused gels, again demonstrating the importance of intact vasculature for tissue functionality.[141]

Induced organogenesis

Induced organogenesis is a completely new approach that focuses on the generation of functional, implantable organs and offers a possible future option to open up a theoretically indefinite source of (autologous) cells. In a newsworthy experimental study, human induced pluripotent stem cells were differentiated into endodermal cells and co-cultured with human mesenchymal stem cells and hUVECs *in vitro*.[142] After self-organization of these cells into a kind of vascularized hepatic precursor, they were implanted into immune-deficient mice. Interestingly, the authors could demonstrate integration of the *in vitro* generated liver organoid into the vascular system of the recipient only 48 h after implantation. Furthermore, the 'liver-bud' matured to functional tissue, resembling adult liver tissue and was able to rescue mice in a drug-induced lethal liver failure model. These encouraging results open up a new field for further research and demonstrate that experimental mimicking of organogenesis might lead to liver support therapies in the future.

Conclusions

Although orthotopic whole liver transplantation is the gold standard for the treatment of irreversible liver failure, the dramatic shortage of donor organs suitable for transplantation has led to alternative strategies, such as the use of marginal organs, donation after cardiac death and living-donor liver transplantation, with considerable risk for the recipient (and in the case of living donor liver transplantation, for the healthy donor). In this desperate situation, alternative concepts to whole organ transplantation are urgently needed. Artificial and bioartificial liver support have been investigated for more than three decades; however, the clinical results are by far not comparable to those of whole liver transplantation. Whereas the detoxification by unselective adsorption of (albumin-bound) blood components by artificial devices seems to be insufficient to support a sophisticated process such as the regeneration of a failing liver, the clinical impact of bioartificial devices is mainly limited by a conceptual issue: their membranes.

Thus, new approaches enabling direct contact of cells and blood were and are constantly being developed to overcome the limitations of current extracorporeal liver

support devices; these approaches include hepatocyte transplantation and tissue engineering concepts such as the repopulation of decellularized livers and organ printing. However, a reliable source of safe and metabolically active cells in adequate numbers to treat patients, their vascularization and (at least temporary) integration into the host circulation so far are the main limiting factors for all liver support approaches. The application of tumour-derived cell lines (for example, C3A or HepG2) implicates the risk of metastatic spreading, and pig hepatocytes are highly immunogenic and a potential route for xeno-zoonoses. Consequently, the directed differentiation of induced pluripotent stem cells might emerge as a future solution, if expansion and differentiation techniques become technically and economically feasible on a clinically relevant scale without risks for the recipients.

Hepatocyte transplantation has the potential to overcome the technical limitation of bioartificial liver support, as the transplanted cells directly integrate into the recipient's body. Moreover, as one donor liver can provide cells for multiple patients, hepatocyte transplantation can help reduce the urgent scarcity of donor organs. Successful hepatocyte applications have been reported for treatment of distinct metabolic liver disorders and clinical studies are ongoing to further evaluate this indication. As the results of clinical hepatocyte transplantation for acute or chronic liver failure are not yet convincing, new concepts are currently being developed to bring hepatocyte transplantation forward toward these indications.

Tissue engineering concepts are still at an early stage of development but are being studied intensively. The repopulation of decellularized organs might have the quickest chance of translation into the clinic, but implantations of recellularized livers will be impossible until vascular integrity (that is, 're-epithelialization') can be re-established or thrombogenesis within these organoids can be prevented without deleterious systemic effects. Furthermore, intensive preclinical research is needed to investigate the complex interactions of decellularized matrices and the cells equipped for repopulation to prevent patients from potential unrecognized long-term risks.

The accurate placement of cells, biomaterials and other components (such as growth factors, drugs and toxins) by rapid prototyping techniques (for example, organ printing) is a thrilling approach and holds immense potential for complex hepatic *in vitro* models as well as for a further decoding of our understanding of tissue and organs. However, integration of these techniques into clinical settings seems to be limited until issues of scalability and quicker maturation are solved. Directed organogenesis of stem and progenitor cells in settings of combined *in vitro* and *in vivo* tissue engineering will be the object of further research and seems promising to enable novel liver support therapies in the future.

Review criteria

The present Review is based on the personal experience of the authors and an extensive review of the existing literature. This literature includes PubMed listed articles and reviews, as well as ongoing studies listed on http://www.clinicaltrials.gov. The search terms for the PubMed searches included the following: "artificial liver", "bioartificial liver", "bioreactor AND liver", "extracorporeal liver support", "hepatocyte transplantation AND human", "liver cell transplantation AND human", "decellularization AND liver", "recellularization AND liver", "ECM AND liver", and "organ printing".

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Author contributions

All authors contributed equally to all aspects of this manuscript.