



Normothermic liver machine perfusion as a dynamic platform for regenerative purposes: What does the future have in store for us?

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Summary

Liver transplantation has become an immense success; nevertheless, far more recipients are registered on waiting lists than there are available donor livers for transplantation. High-risk, extended criteria donor livers are increasingly used to reduce the discrepancy between organ demand and supply. Especially for high-risk livers, dynamic preservation using machine perfusion can decrease post-transplantation complications and may increase donor liver utilisation by improving graft quality and enabling viability testing before transplantation. To further increase the availability of donor livers suitable for transplantation, new strategies are required that make it possible to use organs that are initially too damaged to be transplanted. With the current progress in experimental liver transplantation research, (long-term) normothermic machine perfusion may be used in the future as a dynamic platform for regenerative medicine approaches, enabling repair and regeneration of injured donor livers. Currently explored therapeutics such as defatting cocktails, RNA interference, senolytics, and stem cell therapy may assist in the repair and/or regeneration of injured livers before transplantation. This review will provide a forecast of the future utility of normothermic machine perfusion in decreasing the imbalance between donor liver demand and supply by enabling the repair and regeneration of damaged donor livers.

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Introduction

Liver transplantation offers the only definite cure for end-stage liver disease but primarily relies on the supply of suitable donor livers. Because of the ongoing imbalance between demand and supply, up to 20% of patients die while on the transplant waiting list.¹ The discrepancy between organ availability and demand has forced an increasing use of "extended criteria donor" (ECD) livers for transplantation, including fatty livers, livers from donors of older age, or livers donated after circulatory death (DCD). However, a significant number of ECD livers are currently discarded.^{2,3} Meanwhile, fatty liver disease has emerged as the most common chronic liver disease, affecting up to a quarter of the global population. It is expected that fatty liver-induced cirrhosis will become the leading indication for liver transplantation worldwide.^{4,5} Besides, due to the success of transplant oncology, indications for liver transplantation are expanding.^{6,7}

A practical solution to this conundrum is *ex situ* dynamic preservation of livers before transplantation to aid in the careful selection of ECD livers, thereby optimising utilisation rates. Also, *ex situ* dynamic preservation has shown that many livers that were initially discarded can be safely used, leading to the extension of standard criteria for donor livers. Compared to traditional static cold preservation, *ex situ* machine perfusion improves the quality of donor livers, and allows for longer

preservation times and viability testing.^{7–11} By using normothermic (37°C) machine perfusion (NMP), high-risk ECD livers have been successfully transplanted with excellent short-term outcomes.^{7,10,12,13} Several clinical trials have already been conducted, and many more are ongoing, primarily to investigate the feasibility of NMP, patient and graft survival, postoperative and graft complications, and to perform graft viability assessments. A detailed overview of different (ongoing) clinical trials is presented elsewhere.^{14,15} However, to further increase the availability of donor livers suitable for transplantation, new strategies are required that make it possible to use organs that are initially too damaged to be transplanted. With the current progress in NMP research, whereby experimental human donor livers have been preserved for up to 7 days on a custom-made NMP device,¹⁶ NMP may become a platform for regenerative medicine approaches, enabling repair and reconditioning of injured donor livers, immunomodulation, and other treatments in the future.

This review provides a forecast of the potential future utility of long-term (≥ 24 h) NMP in decreasing the discrepancy between donor liver demand and supply (Fig. 1 and 2). The use of NMP outside liver transplantation, for instance as a platform to test the safety and effectiveness of new therapeutics in metabolically active livers before

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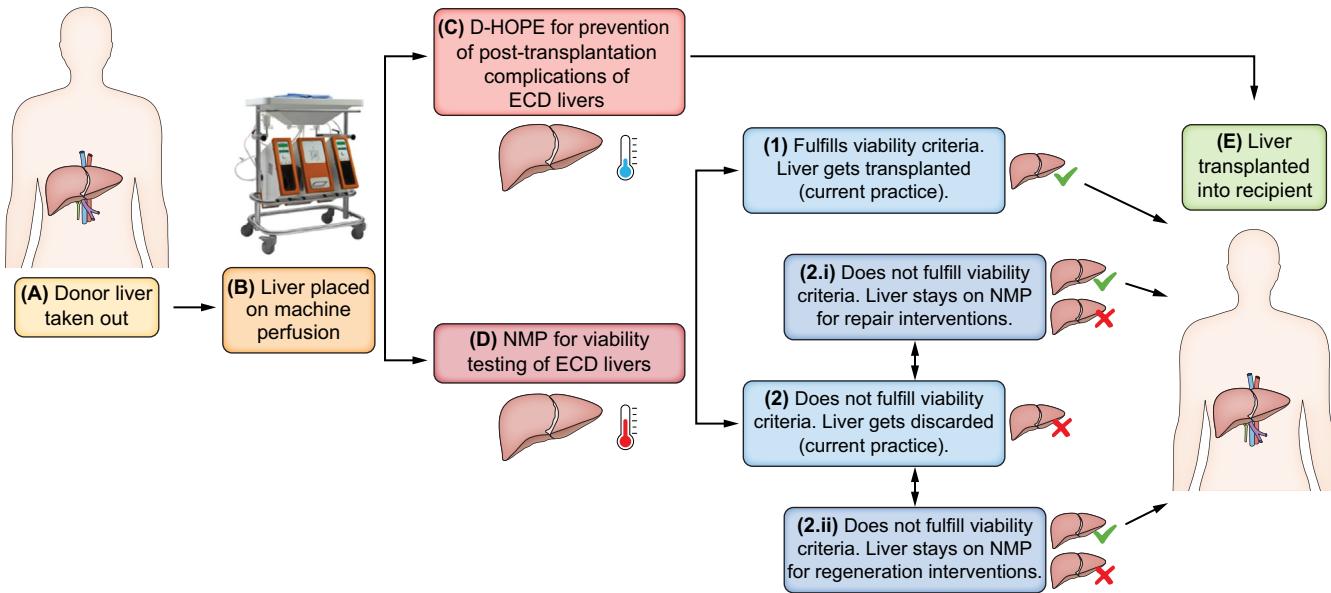


Fig. 1. NMP as a platform for repair and regenerative medicine purposes. To obtain a better balance between the supply and demand for donor livers, NMP can be used as a platform for repair and regenerative medicine to improve the quality of extended criteria donors and make them suitable for transplantation. (A) Donor liver taken out. The liver can be damaged during life, procurement, and/or preservation. (B) The liver is placed on machine perfusion after procurement. Machine perfusion can be used for several purposes, depending on the (expected) degree of injury to the donor liver and/or logistical circumstances. (C) D-HOPE: livers from DCD donors can be placed on hypothermic perfusion to decrease post-transplantation complications (e.g., due to IRI). This is already implemented in clinical care in some clinics. (D) NMP: the viability of high risk, ECD livers of questionable quality can be tested with NMP: i) When the liver fulfils the viability criteria, the liver proceeds to transplantation (current practice). ii) When the viability criteria are not met, the liver will be discarded (current practice). The liver could remain on NMP for treatment to repair or regenerate it in the future. iii) When viability criteria are not met, the donor liver remains on NMP for therapeutic interventions. Different treatments (e.g., defatting cocktails, RNAi, senolytics) can be used during NMP for donor livers that are too damaged for transplantation. Because during NMP the liver is perfused in isolation, the therapeutics will only affect the liver and no other parts of the human, reducing potential side effects and decreasing the amount of drug needed, and thus the cost. When the liver subsequently meets the viability criteria after the interventions, the repaired donor liver proceeds to transplantation. When criteria are not met, the liver will be discarded. iv) When criteria are not met, the liver remains on NMP for the regeneration interventions. Regeneration can take place with decellularised scaffolds and/or organoids. Also, NMP can be used for the regeneration of partial livers. Livers from deceased donors can be split into 2 parts, or partial livers from living donors can be placed on NMP for regeneration to a full size, passively or with treatment (not shown). (E) Livers that were initially discarded are now transplanted after the use of (long-term) NMP. DCD, donation after circulatory death; D-HOPE, dual hypothermic oxygenated machine perfusion; ECD, extended criteria donor; NMP, normothermic machine perfusion; RNAi, RNA interference.

the start of clinical trials, is outside the scope of this review.

old/damaged tissue to regenerate damaged livers. This review will adhere to the aforementioned terminology for repair and regeneration.

Definitions for liver repair and regeneration

Although organ repair and regeneration are commonly used terms in the field of machine perfusion, there is currently no consensus about their exact definition. In a review, Resch *et al.* did not find uniform terminology and concluded that only the term organ preservation should be used for now because organ reconditioning, repair, and regeneration have not yet been established clinically.¹⁷

According to the Cambridge dictionary, repair means: "to put something that is damaged, broken, or not working correctly, back into good condition or make it work again".¹⁸ This would mean that repairing damaged livers is to correct the damage that has occurred during the donor's life, the procurement, or the preservation period. For regeneration, the Cambridge dictionary definition in biology is: "to grow again, or to make something grow again, for example, new tissue or a new part".¹⁹ This would imply that it is necessary to renew and/or replace the

NMP as a platform for repair and regeneration

Promising therapeutics to treat ECD livers are defatting strategies, RNA interference (RNAi), and senolytics. These therapies mainly focus on repairing the damage that has occurred to the donor liver during life (e.g. steatosis, old age) or during procurement and/or reperfusion (e.g. DCD). When donor livers are too damaged, and repair during NMP is not possible or desirable, assisted regeneration of a part of the liver (e.g. the bile ducts) or the whole liver could be an alternative. The use of regeneration with autologous cells from the recipient can also minimise the complications of rejection and the use of immunosuppressants.^{20,21} In deceased donor liver transplantation, it is currently not possible to use autologous cells because of the limited time between the procurement of the liver allograft and subsequent transplantation. However, with the advent of long-term

Key point

High-risk donor livers are increasingly being used to reduce the discrepancy between organ supply and demand.

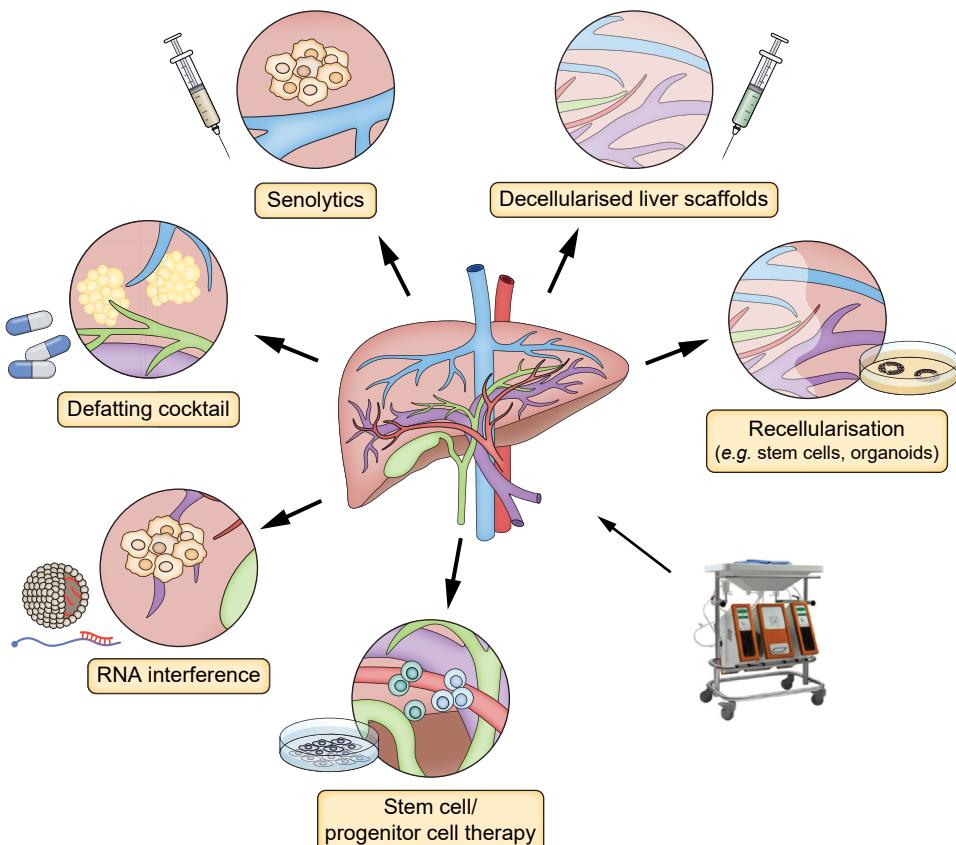


Fig. 2. Regenerative medicine possibilities for the liver during NMP. We envision that any donor liver that is too injured for transplantation could be repaired or regenerated during NMP using several regenerative medicine techniques, such as defatting cocktails, senolytics, RNA interference, stem cell, and progenitor cell therapy, and decellularising drugs to obtain functional liver scaffolds for recellularisation, for example with organoids. These therapeutics can be added to the perfusate solution or injected directly into the liver or vessels. The continuous blood/perfusate flow creates an optimal environment for the therapeutics to work and provide oxygen and nutrients for the liver. NMP, normothermic machine perfusion.

NMP, this might become a possibility. Whole liver regeneration of large animals or humans has not yet been accomplished, but rapid developments in whole organ decellularisation, generation of scaffolds, and creation of human organoids, in combination with long-term NMP, may create a range of possibilities.

Also, after a reduction (e.g. after partial hepatectomy or liver split) in mass, the liver has the ability to regenerate almost all of its original mass within a week.^{22,23} Mueller *et al.* have shown that long-term perfusion of partial human livers is possible, which could allow for the use of isolated perfused hemi-livers in the future.²⁴

Conditions to support liver repair and regeneration

In clinical settings, NMP was initially used for a duration of 3–6 h, but (experimental) perfusion of 24 h and longer is not uncommon anymore.^{16,25,26} However, to support liver repair and mainly regeneration, long-term NMP (>24 h) will be necessary. To keep a liver metabolically active for

more than 24 h, the machine has to mimic the human body to maintain a physiologic environment for the liver (Fig. 3). However, several developments are required to make long-term NMP a success. These include long-term oxygenators that last for at least 7 days, pumps that minimise haemolysis, an artificial kidney to remove waste products, an artificial pancreas and nutrients to meet the demands of the liver, a container where the liver stays undamaged and sterile, and a perfusate containing an oxygen carrier, but also medication to prevent or limit activation of coagulation and the immune system.²⁷ As Eshmuminov *et al.* showed in their research on 7-day *ex situ* liver NMP, 5 major obstacles needed to be addressed by them: i) control of glucose metabolism, ii) prevention of haemolysis, iii) removal of waste products, iv) control of perfusate oxygenation, and v) simulation of diaphragm movement to prevent pressure necrosis.¹⁶ Also, sterility was a problem that needed to be addressed.²⁸ Nevertheless, these investigators succeeded in long-term NMP of porcine and human (discarded) livers for up to 7

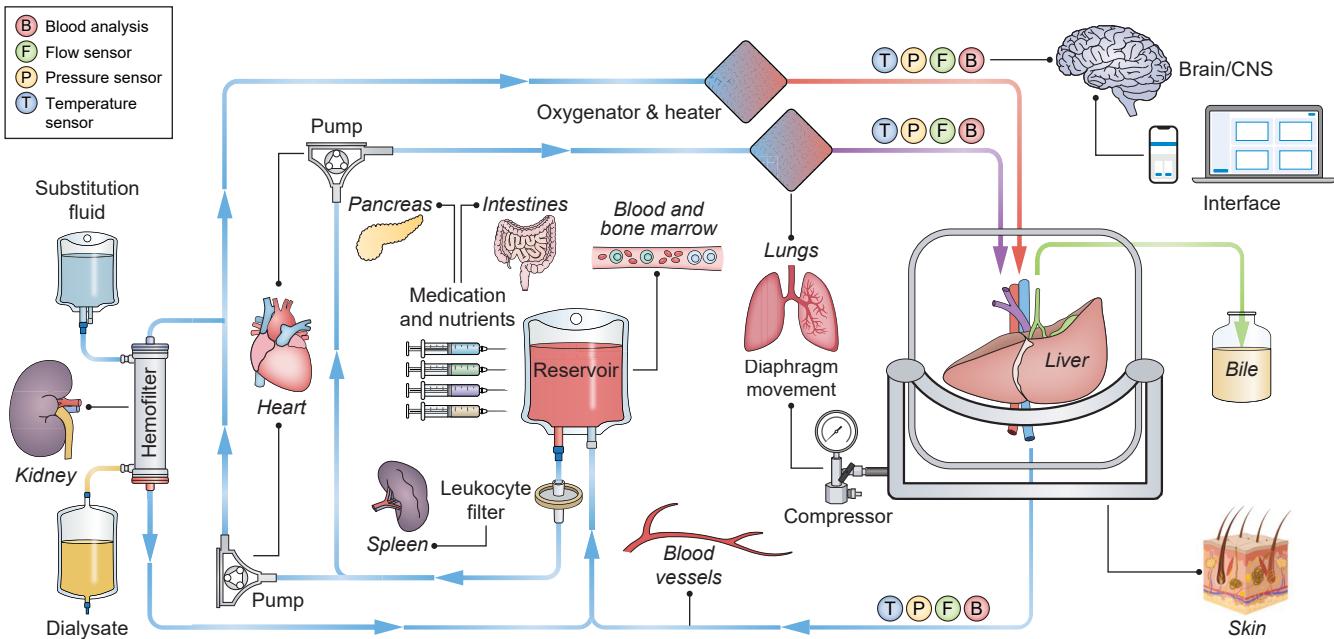


Fig. 3. Graphic presentation of a system for long-term liver NMP. Graphical presentation of an example of a machine that will make long-term (>24 h) normothermic perfusion possible. The machine has to mimic the human body by integrating multiple core physiological functions. The machine will need pumps to mimic the heart to provide controllable blood flow and pressure and an oxygenator for oxygenation and CO₂ removal to replace the lungs. Hormones and nutrients, usually provided by the intestines and pancreas, will be supplemented into the perfusate. It will also need an artificial kidney to remove waste products and a leukocyte filter, mimicking the spleen, for “washing” the blood. The container (skin and abdominal cavity) will provide a sterile environment with an integrated diaphragm movement to help avoid pressure necrosis. Finally, the machine must be automated and monitored from a distance with sensors providing information about the pressure, flow, temperature, and perfusion solution’s composition to maintain a physiological environment. NMP, normothermic machine perfusion.

days, which shows the potential for repair and regeneration of discarded donor livers in combination with NMP.

NMP may also have limitations, such as inducing ischaemia-reperfusion injury (IRI) while the liver perfuses on the machine.²⁹ Therefore it may be helpful to combine NMP with other machine perfusion techniques. Several groups, including the Birmingham group and our group, have applied hypothermic machine perfusion before NMP.^{12,30} This combined approach may lead to improved mitochondrial function during hypothermic machine perfusion,³¹ resulting in reduced oxidative and inflammatory damage during subsequent NMP.³⁰ Especially for long-term NMP, hypothermic machine perfusion prior to NMP might be of particular value.

Ex situ treatment strategies

Defatting strategies

Hepatic steatosis can be classified as mild (<30%), moderate (30–60%), or severe (>60%), and it is often subcategorised as macro- and microvesicular steatosis.³² Severely steatotic livers are generally not used for transplantation because of the increased risk for IRI and primary non-function.³² Several defatting strategies have been studied which might decrease the amount of hepatic steatosis. Some promising results have already been achieved in isolated hepatocytes and precision-cut liver slices,

as well as in rat and discarded human livers in combination with NMP.

An often studied defatting cocktail is the combination of forskolin, scopolamine, nuclear receptor ligands GW7647 and GW501516, hypericin, and visfatin, which all play a role in fat metabolism, uptake, or accumulation.³³ This cocktail led to a 4-fold faster decrease in macrovesicular steatosis in isolated rat hepatocytes and a triglyceride content reduction of 65% in rat livers after only 3 h NMP, compared to a 30% reduction with a control perfusate.^{34,35} Histology showed a significant decrease in lipid vesicles in hepatocytes, especially in the periportal area (zone 1), and a restored hepatocellular cytoplasmic volume.³⁵ The addition of L-carnitine to this cocktail led to even better results, by increasing fatty acid transportation to the mitochondria.^{33,36} Reductions in tissue triglyceride levels and macrovesicular steatosis were seen in discarded human steatotic livers after 6 h NMP. The addition of L-carnitine also decreased IRI, expression of oxidative injury markers, activation of immune cells, and inflammatory cytokines in the perfusate.³⁷

The defatting combination of L-carnitine and exendin-4 led to an increased triglyceride concentration in the perfusate in human steatotic livers after 8 h NMP, but information on changes in tissue fat content was not reported.³⁸ However, these promising results with NMP might also reflect the

Key point

To allow for safe transplantation of high-risk donor livers, NMP enables *ex situ* viability assessment.

effect of a continuous flow passing the hepatocytes. Yarmush *et al.* showed that flow is an important catalyst for the effectiveness of a defatting cocktail. By adding flow conditions to a defatting cocktail in isolated hepatocytes, the defatting time decreased from 48 h to 4–6 h (70% defatting), probably because of the increase in L-carnitine uptake by the cells.³⁶ Another possibility to increase the speed of defatting passively might be the use of mild hyperthermia during machine perfusion.³⁹

Unfortunately, some of these compounds are not approved for clinical use yet and might actually be harmful despite these encouraging results. The first mentioned defatting cocktail has been reported to increase lactic dehydrogenase activity, and the GW-compounds are associated with hepatic carcinogenesis and mitochondrial dysfunction.^{33,40} For this reason, other agents have also been tested, with success. The replacement of the GW-compounds with 2 polyphenols, epigallocatechin-3-gallate, and resveratrol, led to less hepatotoxicity in rat livers, and glial cell line-derived neurotrophic factor led to no alteration in lactic dehydrogenase activity in steatotic mouse livers after 4 h NMP, while both were still effective.^{33,40}

In summary, in experimental studies, defatting cocktails can decrease steatosis in livers in just a few hours. However, with long-term NMP, it might become possible to defat livers without extra medication because a “spontaneous” decrease in hepatic triglyceride content and steatosis is also seen without defatting cocktails.^{35,41} To make this work, we propose that the fat that is transported to the perfusion solution (e.g., as very-low-density lipoprotein) should be removed from the perfuse with a fat filter. Otherwise, the perfuse will become saturated with fat, a defatting limit will be reached, and potentially, fat emboli may occlude the oxygenators leading to impaired gas exchange.

RNA interference

RNAi is a natural process of post-transcriptional gene regulation that inhibits the translation of mRNA into proteins by adding a complementary strand. By explicitly targeting genes related to the causes of donor liver damage or post-transplantation complications, such as IRI or graft rejection, these genes can be transiently silenced to prevent such problems from arising.⁴² Several molecules, including micro-RNA, small interfering RNA (siRNA), and short-hairpin RNA, can be used for therapeutic RNAi.⁴²

IRI is associated with liver apoptosis, mediated by death receptors such as Fas and tumour necrosis factor α , and mitochondrial dysfunction induced by cellular stress. Also, apoptotic genes, such as caspase-8 and caspase-3, and those involved in the nuclear factor- κ B pathways can induce IRI.^{43–45} Silencing these genes in rodent liver and transplantation models of IRI has resulted in less Fas, caspase-8, and caspase-3 protein expression and activity and better preservation of the liver architecture, indicating

diminishing IRI.^{43,44} Many more experiments have been successfully performed on different targets to diminish IRI and graft rejection, as reviewed by Brüggenwirth *et al.*⁴²

The downside of these agents is that they have to be administrated to the donor a few hours to days before organ procurement.⁴² This is where NMP may become an attractive solution. During NMP, the medication to target these genes can be administrated *ex situ* to the isolated liver before transplantation. This has already been tested in a few experimental studies.^{46–49} siRNA against the Fas receptor, coated with invivofectamine lipid nanoparticles, were added directly to the perfusion solution and were taken up by hepatocytes in a rat liver after 4 h NMP.⁴⁶ Ablation of endothelial cell class II major histocompatibility complex molecules, which can reduce protein expression in the allograft and protect the graft against rejection, has been achieved by administering siRNA-releasing poly(amine-co-ester) nanoparticles during NMP – this led to effective and reliable particle uptake and major histocompatibility complex class II molecule silencing in vascular endothelial cells in mouse livers, without causing toxic effects.⁴⁸

RNAi can also be used for antiviral treatment of the donor liver, such as for hepatitis C. The hepatitis C virus is dependent on the presence of microRNA-122, which can be silenced by miravirsen, a locked-nucleic acid oligonucleotide that can inhibit hepatitis C virus replication. After 4 h of NMP, miravirsen led to microRNA-122 sequestration and target gene derepression in porcine livers, preventing hepatitis C infection after liver transplantation.⁴⁹

The success of these experiments can be enhanced using *ex situ* NMP, which allows for the delivery of therapeutics into the isolated liver. This will minimise systemic toxicity and reduce costs, as only the dose required for a single organ will be needed. In this way, RNAi can directly act inside the liver, thereby potentially curing, protecting, or repairing damaged livers before transplantation. While the current literature on the application of RNAi during NMP is limited to experimental studies, RNAi in other fields is already being explored in clinical trials.⁵⁰ The application of RNAi in liver transplantation is still in its infancy, but successes in other areas make RNAi a serious candidate to become a therapeutic agent for use during NMP in the future.

Senolytics

Senescent cells (SCs) are cells in an irreversible state of cell-cycle arrest that triggers the production of a range of inflammatory cytokines and chemokines, matrix metalloproteinases, and growth factors referred to as the senescence-associated secretory phenotype.^{51,52} Cellular senescence results from a stress response, such as inflammatory or metabolic signals, DNA damage, reactive oxygen species, and mitochondrial dysfunction.⁵¹ For this reason, SCs play an important role in age-related and chronic

Key point

NMP also provides an optimal platform for regenerative medicine purposes because of oxygen and nutrient delivery.

diseases, as well as in solid organ transplantation due to the systemic response to IRI.^{51,53} Thus, targeting SCs to limit the senescence-associated secretory phenotype during and shortly after liver transplantation is garnering interest.

Senolytics are a group of drugs that selectively target SCs to remove their protection from apoptosis and hence induce apoptosis.⁵³ Because SCs can form from all kinds of cells, different senolytics can target specific tissue types. Several different senolytics are currently being investigated, and some are already being tested in clinical trials, such as dasatinib and quercetin, navitoclax, and heat-shock protein 90 inhibitors.⁵¹

Senescence plays a major role in liver dysfunction. Accumulation of SCs promotes hepatic fat accumulation and steatosis, which were significantly reduced after dasatinib administration in mouse livers and human adipose tissue.^{54,55} The same treatment also cleared SCs and diminished cell-free mitochondrial DNA release in old mice, thereby dampening the senescence-associated secretory phenotype and prolonging the survival of old cardiac allografts.⁵⁶ Furthermore, the treatment with the senescence-inhibiting drug ABT-737 improved regeneration by disrupting p21 expression, a senescence-associated gene.⁵⁷

Besides hepatocytes, cholangiocytes are also influenced by SCs. Senolytics, such as B-cell lymphoma-extra-large inhibitors (A-1331852 and navitoclax) that induce apoptosis in senescent murine biliary epithelial cells or AP20187 and fisetin that reduce senescence markers associated with cholangiopathy could be used to treat cholangiopathy.^{58,59}

Senolytics can potentially be administrated to the donor or the recipient. However, during NMP, senolytics will directly target the isolated liver, thereby enabling optimal dosing to achieve adequate treatment effects and reduce the risk of side effects. To date, no experimental or clinical research is available on the combination of senolytics with NMP; future work will need to demonstrate its efficacy to diminish or prevent post-transplantation complications, such as post-transplant cholangiopathy.

Stem cell/progenitor cell therapy

Stem cells are cells with the ability to divide for an indefinite period and can differentiate into different kinds of cells under specific conditions and signals.²⁰ Mesenchymal stem cells (MSCs) are present in various adult organs and often function to support organs. MSCs can be isolated from various tissues and organs, such as bone marrow and adipose tissue, and may be used to treat diseases. MSCs have immunosuppressive functions, participate in the anti-inflammatory response, and secrete cytokines that inhibit the macrophage-mediated inflammatory response and reduce hepatic IRI because of their reparative immunomodulatory effects.^{60–62}

MSCs are most often used in experimental settings to reduce IRI and to overcome rejection via their immunomodulatory action.⁶³ In a preclinical setting with rats, injection of MSCs after reperfusion preserved hepatocyte integrity and suppressed inflammatory responses, oxidative stress, and apoptosis, and inhibited acute allograft rejection after liver transplantation.^{62,64–66}

Some studies showed promising results with MSCs in combination with machine perfusion. Human bone marrow MSCs (BMMSCs) have been injected into the hepatic artery or portal vein of a porcine liver kept on hypothermic machine perfusion for 30 min, where a wide range and patchy distribution was shown, with preserved paracrine activity. After 4 h of NMP, to mimic the reperfusion phase of liver transplantation, an increase in the cytokines IL-6 and IL-8 was observed, showing regenerative and immunomodulatory effects.⁶⁷ Injection of BMMSCs during NMP of DCD rat livers led to improved liver function with reduced histological damage, serum and liver pro-inflammatory cytokine levels, oxidative stress injury, mitochondrial damage and biliary epithelial cell injury, and prolonged survival time.^{60,68–70}

These improvements were enhanced by administering heme oxygenase-1-modified BMMSCs during NMP in rats, which improved post-transplant survival⁷¹ and promoted activation of peribiliary glands to facilitate repair of injured bile duct epithelium in recipient rats.⁷² These promising results were also seen in discarded human livers. Multi-potent adult progenitor cells were infused directly into the right lobe via the right hepatic artery or the portal vein during NMP. The introduction of the cells directly into the target organ was successful, without any noticeable adverse effect on the perfusion itself.⁷³

Extracellular vesicles (EVs) released by stem cells have also been used in a preclinical setting to mitigate IRI.^{61,74,75} EVs play an important role in cell-to-cell communication and contain several materials, such as proteins, lipids, and RNA, that can transfer to other cells.⁷⁶ Human liver stem cell-derived EVs were added during NMP of rat livers. After 4 h of NMP, EV uptake by hepatocytes was shown, and the rat liver allograft showed less histological damage and injury markers.⁷⁴

Regeneration techniques

Even though a liver itself has regenerative properties, for assisted regeneration, a good basic structure that can provide biomechanical support for tissue to regenerate is preferred. A scaffold, which is a template on which (stem) cells can be attached to proliferate, differentiate, migrate, repair, and regenerate new tissue, is frequently used for this purpose.^{21,77,78} Because scaffolds have to mimic the properties of the extracellular matrix (ECM), regarding both structure and cellular behaviour/interactions, scaffolds made by decellularisation of natural tissues or organs have

Key point

Regenerative therapies delivered during NMP may rescue donor livers that currently cannot be transplanted.

many advantages, such as maintaining natural geometric morphology, vasculature structure, and active ECM proteins that are beneficial for recellularisation.^{77–83} The downside is the need for (discarded) organ donors. Although porcine livers might also be used for decellularisation and recellularisation with human cells in the future.^{84,85}

Different protocols can achieve decellularisation, including chemical and enzymatic reagents and physical methods.^{77,81,86,87} For whole organs, the perfusion method is preferred and is possible because the basic structure of the vascular system stays intact. This method shows better preservation of the ECM and has the advantage of supplying the liver with oxygen and nutrients.^{79,81,88} Besides whole livers, bile ducts can also be decellularised to serve as a scaffold.⁸⁹

While liver decellularisation is a delicate procedure, promising experimental results have already been reported with animal and discarded human livers. The basic architecture of the liver allograft, consisting of the ECM with the biliary drainage network and the vascular structure, is preserved and immunological compounds are removed.^{80,82,86–88,90–95}

After decellularisation, recellularisation has to take place. Rebuilding the scaffold can be achieved by several cells. Induced pluripotent stem cells (iPSCs) are most often used, but hepatic and endothelial progenitor cells, and embryonic, foetal and mesenchymal stem cells have also been used, as have foetal and adult/mature hepatocytes.^{80,92,93,96–98} Because the liver is composed of many different cell types, it is a challenging task to rebuild a whole functioning liver. Approximately 30 billion hepatocytes are required for a full-size liver, which is not easily achieved.^{78,80,99} The sheer number of cells required is not the only problem, which cell types to use, the method, sequence/timing of seeding, and the culture media also have to be studied before whole livers can be regenerated.^{21,96,100}

Different recellularisation methods have been used, but the continuous perfusion method has shown many advantages and is now commonly used.⁹⁶ During machine perfusion, cells are either injected into the perfusion fluid or directly into the portal vein in a single or multi-step approach, with the multi-step approach leading to a higher number of cells attaching to the liver.^{80,92,93,96,101} The machine perfusion method also provides a better distribution of cells. One study showed that the primary rat hepatocytes remained around the vessels for the first 4 h, but after 1–2 days, they were distributed to the whole decellularised liver scaffold.⁸⁰ Also, the direction in which the seeding occurs can have an influence on the distribution of cells, which is better with antegrade flow (through the portal vein) than with retrograde flow (through the vena cava and hepatic veins).¹⁰²

Machine perfusion already plays an important part in this method of experimental liver

regeneration. For decellularisation, either hypothermic machine perfusion alone or combined with NMP can be used, but for the regeneration process, NMP is more appropriate.^{80,87,90,93} This method of regeneration can not only be used for the generation of whole livers but can also be used as a therapeutic option for the administration of liver cubes in hepatic diseases or after hepatectomy.^{86,103} However, despite major progress in this field, no large animal or human livers have been regenerated and transplanted yet. This is mainly because of the diversity and very high number of different cells that need to be generated in line with good manufacturing practices and the difficulties of re-endothelialisation of the vascular system to prevent coagulation activation.⁹⁶

The development of organoids has been increasing over the last decade. An organoid has recently been defined by consensus as a “three-dimensional structure derived from (pluripotent) stem cells, progenitor, and/or differentiated cells that self-organize through cell-cell and cell-matrix interactions to recapitulate aspects of the native tissue architecture and function *in vitro*”.¹⁰⁴ Liver organoids can be developed from the same cells used to recellularise scaffolds. The cells can differentiate into different cells depending on the culture medium and conditions used.^{104–107} Also, autologous cells can be obtained from patients to decrease graft rejection and immunological problems after transplantation.⁸⁹ However, primary hepatocytes remain scarce, which is why iPSCs are still commonly used.¹⁰⁵

The advantage of a 3D structure is that organoids resemble the actual tissue better, including the architecture and ECM, which is important for cell-cell interactions.^{78,81,83} However, a problem with large 3D organoids is the vascularisation of these structures and not receiving enough oxygen and nutrients, resulting in necrosis. This problem might be solved by NMP, where the perfusion machine acts as a platform for the implantation and distribution of organoids in injured liver allografts. In an experimental setting, Sampaziotis *et al.* have used NMP to deliver cholangiocyte organoids to donor livers and demonstrated repair of injured bile ducts. Cholangiocyte organoids, made from primary human cholangiocytes, exhibit plasticity and can keep their *in vivo* signatures when placed around the “original” biliary tree. The organoids were injected into a terminal branch of the intrahepatic ducts. After only 100 h of NMP, the organoids were still in place and had been regenerating so that after NMP, the intrahepatic ducts consisted of native and transplanted cells, without signs of cholangiopathy and with higher pH and higher bile volume than control livers.¹⁰⁸ This success represents an early example of the increasing possibilities of long-term NMP in the development of organoids and the regeneration of damaged livers.

Key point

Major challenges and barriers still need to be overcome before NMP can be used for this purpose.

Table 1. Proposed research agenda to enable repair and regeneration of damaged livers with NMP in the future.

Subject	What remains to be done?
Long-term (≥ 24 h) NMP machine	Assessment of safety, feasibility and efficacy, and obtaining clinical approval for long-term NMP devices
Trained personnel	Development of clinical training programs
<i>Ex situ</i> treatment strategies	Assessment of safety, feasibility and efficacy, and obtaining clinical approval
Viability criteria	Validation of previously established viability criteria
Costs	Development of new viability criteria
	Economic cost-utility analysis of new treatments and technology

NMP, normothermic machine perfusion.

Challenges and barriers

The aforementioned future ideas for possible treatments and therapies to treat damaged livers with NMP can only become a reality if more research is done and some major challenges and barriers are overcome.

A major barrier is having clinically certified long-term NMP devices available to apply these treatments. Although no such machine is currently available, both clinically certified short-term machines as well as custom-made devices are used for perfusion for up to 7 days for experimental research.^{16,26,109} In addition to most of the treatments described, devices that enable long-term

NMP are not yet clinically approved, which could potentially take several years.

Another challenge is the translation of these treatments with (long-term) NMP into clinical practice. Even if an NMP machine becomes fully automated, it will require trained personnel who will need to be able to adjust the machine at any time of day as needed. The NMP device must also be readily accessible to certified personnel at all times while maintaining sterility.

For long-term NMP, viability assessment criteria need to be established to evaluate treatments and decide whether the liver is suitable for transplantation or not. Currently, (non-validated) hepatocellular and/or cholangiocellular viability markers are used.^{110,111} However, when using NMP for repair and regeneration, new biomarkers will have to be identified to act as viability criteria in order to evaluate the effect of the treatments in addition to the current criteria (Box 1).

Not unimportantly, costs associated with the implementation of long-term NMP should be evaluated, especially in light of exponentially increasing overall healthcare costs. In the end, evidence of the clinical and economic advantages of this new technology will be needed to drive its implementation.¹¹²

Despite an increase in experimental research on repair and regeneration strategies of damaged donor livers in combination with NMP, many challenges and barriers need to be addressed first. Table 1 shows an agenda outlining the topics that need to be explored before the treatments described in this review can become a reality.

Conclusion

To reduce the discrepancy between organ demand and supply in liver transplantation, NMP may increasingly be used in the future as a dynamic platform to optimise organ utilisation by enabling the repair and regeneration of damaged donor livers. Machine preservation has already been shown to be of great value in diminishing post-transplantation complications and increasing the donor pool via increased use of high-risk ECD livers (following pre-transplant viability testing). In the last few years, experimental research on the repair and regeneration of organs has increased exponentially. Several therapeutics, such as defatting cocktails, RNAi, senolytics, and stem cell therapy, might repair damaged livers, whereas stem cell therapy, scaffolds, and organoids may assist in the regeneration of injured livers before transplantation.

Current viability criteria (validated and non-validated) ^[113-115]
<i>Hepatocyte function</i>
Bile production
Injury markers (ALT, AST)
Lactate clearance
pH maintenance
Glucose metabolism
Coagulation factors (D-dimer, platelets)
miRNAs
Inflammation markers (Interleukins)
TNF- α
Endothelin-1
FMN
<i>Cholangiocyte function</i>
Biliary pH, bile/perfusate ratio
Biliary glucose, bile/perfusate ratio
Biliary bicarbonate, bile/perfusate ratio
Biliary LDH
Cholangiocyte-derived microRNAs-122
<i>Long-term NMP viability criteria (additional to current)^[16, 116, 117]</i>
Response to vasoactive agents
Response to pancreatic hormones
Production of complement factors
Production of coagulation proteins
<i>Treatment specific (additional to current and long-term)</i>
Triglyceride content (defatting cocktails)
Protein expression (RNAi, stem cell/progenitor cell)
Senescence markers (senolytics)
Biopsies (regeneration, RNAi, stem cell/progenitor cell)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FMN, flavin mononucleotide; LDH, lactate dehydrogenase; miRNA, microRNA; NMP, normothermic machine perfusion; RNAi, RNA interference; TNF- α , tumour necrosis factor- α .^[113-117]

Nevertheless, some major challenges and barriers need to be overcome before NMP can be used as a platform for the repair and regeneration of damaged donor livers. Currently, only a minority of liver transplant centres worldwide have employed NMP in their clinical practice. If future high-level evidence supports the widespread introduction of these techniques, overcoming logistical constraints, including optimal utilisation of these relatively expensive devices and the availability of specially trained perfusion personnel, will be essential. In addition, most of the aforementioned therapies are based on experimental research and require extensive clinical testing and approval before being used in clinical practice. This review may guide the future research agenda on NMP as a dynamic platform for the repair and regeneration of damaged donor livers, potentially leading to better utilisation of the current donor pool, ultimately reducing transplant waiting lists and improving patient outcomes.

Abbreviations

BMMSCs, bone marrow mesenchymal stem cells; DCD, donation after circulatory death; ECD, extended criteria donor; ECM, extracellular matrix; EVs, extracellular vesicles; iPSCs, induced pluripotent stem cells; IRI, ischaemia-reperfusion injury; MSCs,

mesenchymal stem cells; NMP, normothermic machine perfusion; RNAi, RNA interference; SCs, senescent cells; siRNA, small-interfering RNA.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

Concept and design: B.L., V.E.d.M and R.J.P. Writing of article & preparing figures and tables: B.L. Editing and revision & approval of the final version: B.L., V.E.d.M and R.J.P.

Supplementary data

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Author names in bold designate shared co-first authorship

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