Long-term machine perfusion: a new tool in the box to increase available liver grafts

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Abstract

Liver diseases are becoming more prevalent in the western world. At this moment, the only viable therapy for end-stage liver diseases is liver transplantation. Liver transplantation, as first successfully performed by Dr Thomas Starzl in 1967, has made it possible for a patient with severe end-stage liver disease to recover. However, the need for liver transplantations has been rising over the years, with now over 30.000 transplantations performed worldwide every year. The number of patients requiring transplantation is rising while there are not enough donors, leading to long waiting lists. During the past decades, major advances have been made to fill this gap of available donors. A recent advance is the ability to sustain a liver outside of the body for 7 days. This new technique, long-term machine perfusion, could open up exciting new treatment possibilities: extended duration of long-term machine perfusion could enable genetic modulation on perfused livers, regeneration of partial livers or bioengineering combined with liver perfusion. This review explains how long-term machine perfusion works and discusses the possibilities this technique would enable, looking at already existing techniques in ongoing research and hypothesize how long-term machine perfusion would aid in these techniques.

Layman's summary

Imagine the following: You are diagnosed with a liver disease. And not just any liver disease, you are diagnosed with end-stage liver disease. This means you liver cannot function anymore, and the only relief is the transplantation of a donor liver. Luckily for you, you are alive in a time after 1967, meaning that liver transplantation is clinically possible. Less luckily for you, there are also other people, like yourself, that need a transplant. Transplantation centers all over the world perform over 30.000 liver transplantations in total every year. You, however, are put on a waiting list because there are not enough transplantable livers available.

This review discusses a new technique to shorten the waiting list by making more transplantable livers available, thereby possibly saving your life. The new technique is long-term machine perfusion. In long-term machine perfusion a surgeon and his team remove a liver from a donor and then connect it directly to a perfusion machine. This new machine can keep the liver alive for up to 7 days outside the body.

The low quality of donated livers is one of the reasons of why the waiting list are high. Surgeons do not use these livers because of a too high risk for the patient.
With long-term machine perfusion these low-quality livers could be repaired before they are given to the patient, potentially creating more transplantable livers. This repair could for instance be done with genetic techniques like RNAi and CRISPR-Cas9. The genetic techniques could possibly change animal livers (for instance pig livers) for better xenotransplantation, creating even more available livers.

Splitting healthy donated livers is also used to expand the pool of available livers. Because the liver can regenerate fast, two partial livers can be given to two patients. However, the problem with this is that you give patients suboptimal livers, instead of one good liver. With long-term machine perfusion this problem could be solved by first letting the split livers regenerate outside of the body, before transplanting it into the patient.

Lastly, some researchers look at ways to engineer a liver. They do this by growing the cells of the liver outside of the body, so called organoids (3D cell cultures). Long-term machine perfusion could aid in the engineering of the liver by forming a scaffold to which the organoids can attach.

To conclude, long-term machine perfusion could open up new possibilities for treatment by integrating genetic modulation, regeneration and bioengineering.

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1. Introduction

With approximately 2 million deaths per year worldwide, liver diseases (1.3 million deaths from cirrhosis, 0.6 million deaths from liver cancer) are a big burden on our healthcare system (1). In the last decades, deaths worldwide caused by liver diseases have risen from approximately 1.6 million in 2000 to approximately 2 million in 2019, and this is a rise in the percentage of total deaths from 3,2% to 3,4% (2). Liver transplantation, which was for the first time performed by Thomas Starzl in 1967, has given patients with end-stage liver diseases a second chance (3,4). At this moment transplantation centers all over the world carry out approximately 35.000 transplantations every year, but this is only 10% of the global need (5). There are not enough donors to satiate the need, which leads to long waiting lists (6). Although the mortality rate for patients on the waiting list has gone down since its peak in 2014, the waiting lists are still problematic for patients with a mortality risk of around 12,3 per 1000 waiting list-years (7,8). The shortage of available livers demands for new techniques to bridge the gap between available grafts and recipients.

Transplantation surgeons discard approximately 10% of all donor liver grafts before transplantation because of multiple reasons, mostly related to the low quality of the liver grafts (8). This percentage has gone down through the years until 2018 but is now again rising. Possibly because of a higher percentage of older donors, which gives lower quality grafts (8). The lower quality of the graft could be because of a high degree of steatosis in older liver grafts. This higher steatosis may come from the rising prevalence of obesity in the world, which is one of the high-risk factors in forming steatosis (9,10). Livers recovered from donors after brain death were 4 times less likely to be discarded than livers donated after circulatory death (7,1% vs 29,9%). This has been the same over the last couple of years (8). Discarded grafts are a considerable amount of potentially available liver grafts, especially livers donated after circulatory death. One way in which more of these discarded grafts could still be utilised is with the use of machine perfusion (11,12).Liver machine perfusion on its own can enhance the quality of otherwise discarded livers and can make it possible to monitor the quality of the livers (11,12). Furthermore, new advancements in liver machine perfusion have opened up the possibility for longer *ex vivo* perfusion of liver grafts before transplantation. This longer perfusion duration opens up a window in which otherwise discarded livers or partial livers can be treated *ex vivo* to become suitable for liver transplantation, broadening the pool of available donor grafts (13,14). Three novel techniques have come up in recent years that can be combined with long term liver perfusion.

First, some novel gene-editing techniques have been used in different ways to broaden the pool of available organs. Researchers from the Langone Transplant Institute in New York have used CRISPR-Cas9 to humanize swine organs for transplantation (15,16). In another setting, gene editing was performed on cells *ex vivo* to overcome a genetic liver disease using autologous liver cells, after which the cells were returned to the patient (17). Furthermore, Thijsen et al. recently proposed using RNA interference (RNAi) to help overcome hypoxia-induced damage to liver grafts using machine perfusion (18). Long-term machine perfusion could open up possibilities for similar gene-editing techniques to enhance and repair liver grafts.

Second, the old Greeks with their myth of Prometheus already knew over centuries ago that the liver is an organ with a great regenerative capacity. The liver can fully regain functional mass after injury in a time ranging between 7 days to a couple of weeks (19,20). Several existing techniques already utilise this great regenerative capacity of the liver, for instance, partial liver transplantation and staged hepatectomy of cancerous livers (19,20). Long-term machine perfusion of the liver might open up the possibility of a partial liver to be regrown *ex vivo* prior to transplantation.

Lastly, another approach that has been tried in the last decades to overcome the shortages

of available livers is the use of bioengineering. With bioengineering, the focus is led away from liver grafts obtained via donors and is instead set on making livers from scratch or treating patients with single cells. Techniques like this are for instance cell-based approaches where healthy hepatocytes are given to patients with an unhealthy liver (21). Or instead of single cells, the healthy cells can first form a structure on their own, so-called organoids, and are then given to patients (22–24). A completely new technique is the use of a bioscaffold. One way this can be done is by decellularizing a liver graft so that only the extracellular matrix stays present which could then be repopulated with healthy cells (25). These techniques are all promising but have major disadvantages or a far away from clinical application.

All in all, this review will discuss the possibilities that long-term machine perfusion could enable, looking at genetic modulation, regeneration and finally bioengineering and it will make some hypotheses about how long-term machine perfusion could aid in these techniques.

2. Machine perfusion as a new development in liver graft procurement

This chapter will give a glance at what machine perfusion is and what the benefits are for liver graft procurement. The reason for the introduction of machine perfusion will be given, the different perfusion techniques will be discussed, and the new technique of long-term machine perfusion will be introduced.

2.1 Current practices and problems

Normally, when a liver is obtained from an available donor, the graft is cleaned and then kept on ice. This is called static cold storage (SCS) (26). SCS causes some problems, especially for livers of a lower quality. Not all obtained livers are of a good enough quality, some are of lower quality. In Europe these lower quality livers—which are in literature are also referred to as marginal livers—are assessed with extended criteria and, donors that match these criteria are called extended criteria donors (ECDs). To be named an ECD there needs to be a match with the criteria in table 1 and the liver will be discarded when all criteria are met (27).

Table 1: *Parameters for Extended Criteria Donors. The donor is considered an ECD if it has at least one of these criteria (27).*

Criterium	Value
Donor age	>65 years
Mechanical ventilation time	>7 days
Body mass index (BMI)	>30 kg/m2
% Steatosis	>40 %
Serum sodium concentration	>165 mmol/L
Serum alanine aminotransferase (ALT) concentration	>105 U/L
Serum aspartate aminotransferase (AST) concentration	>90 U/L
Bilirubin concentration	>2.98 mg/dL

The biggest problem encountered with the procurement of both healthy and ECD in SCS is that the liver has a period when there is no blood supply. This phenomenon is called ischemia, and a longer ischemia period could cause ischemia-reperfusion injury (IRI) when the organ is transplanted back to the patient and blood flow is restored (26). The injury is thought to happen because of an accumulation of by-products of anaerobic metabolism (28,29). Because there is no blood flow during ischemia there is also no oxygen going to the organ, causing an accumulation of succinate—a molecule normally used in aerobic respiration. This accumulation causes mitochondrial stress via a molecular cascade (26,28). Interestingly, during the period of ischemia there is no direct damage to the tissue. The damage occurs after oxygen is returned to the organ. When oxygen is returned, reactive oxygen species (ROS) are formed because of the mitochondrial stress that was induced during ischemia. The ROS in turn have downstream detrimental effects (30).

Related to IRI are biliary complications, one of the most prevalent problems with both healthy and ECD liver transplants. Biliary complications are estimated to occur in between 5-32% of all the liver transplant recipients, resulting in a high mortality rate (31). There are three types of biliary complications: biliary leakage, anastomotic strictures (AS) and non-anastomotic biliary strictures (NAS) (31). There is a high rate of re-transplantation associated with biliary problems. It is hypothesised that the epithelial cells of the bile duct are more susceptible to damage from ischemia causing biliary problems and that either circumventing ischemia, or having the reperfusion take place in a colder environment could provide a benefit in this regard (32).

2.2 Normothermic and hypothermic machine perfusion

To overcome IRI, new techniques have been explored and tried in the clinic. The most prevalent of these is machine perfusion of the liver. At this moment there are two major techniques of perfusing the liver: hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) (28,33).

As the name suggests, in hypothermic machine perfusion the liver graft is perfused while it is still in a cold condition. The main form of hypothermic machine perfusion is hypothermic oxygenated machine perfusion (HOPE) (26,32). In HOPE the perfusate is oxygenated and this is thought to reduce hypoxia and mitochondrial energy depletion. There are two forms of HOPE, in normal HOPE the liver is perfused only via the portal vein, and in so-called D-HOPE, there is also perfusion via the hepatic artery. The biggest advantage of HOPE is that there is less damage from reperfusion. The principle behind this is that the temperature of the liver during ischemia matters when the liver is given oxygen after a period of ischemia. The warmer the liver during ischemia, the more mitochondrial stress takes place (32). Normally, with SCS, the liver is perfused and oxygenated normothermically before transplantation, after having been in cold ischemia. This is thought to cause the stress mentioned earlier because of the warm temperature of the oxygenation. However, with HOPE the oxygen reperfusion is also in cold hypothermic conditions, and this has been shown to cause less stress. After this procedure, the graft is heated and given to the recipient in normothermic conditions (26). Essentially you have an extra ex-vivo oxygenation step between the SCS and the transplantation that has shown to significantly reduce side effects. Multiple trials have shown that HOPE has a protective effect on liver grafts compared to SCS (26,34).

Normothermic machine perfusion (NMP) is a perfusion technique where instead of using cold conditions for the perfusion of the organ of interest, the organ is perfused with oxygenated blood or perfusate at body temperature. NMP can be used in two ways: Immediately put the liver graft on the pump after procurement, circumventing the need for SCS. Or, use normothermic machine perfusion after a variable time of SCS. (29). Similarly to hypothermic machine perfusion, NMP has also has a beneficial effect in reducing IRI (29).

One of the advantages of using machine perfusion in general, is that you can monitor the health of the perfused liver. This can be done via monitoring markers in the perfusate to determine the quality of the liver and the amount of potential damage (35). Furthermore, biopsies can be taken from the perfused liver to be used for immunohistochemical analysis (32). For instance, in HOPE it is possible to look at specific markers that indicate if there is ischemic damage present. One marker that can be checked is flavin mononucleotide (FMN), a marker released from complex-I in the mitochondria when damage occurs and is auto-fluorescent so it can be looked at with the use of a spectroscope. NADH levels are also assed in HOPE, indicating a functional liver (26). Furthermore, with normothermic machine perfusion one could assess parameter of cellular reaction that cannot be seen in hypothermic conditions, such as the normal function of hepatocytes. A physician can assess parameters such as glucose levels, bile formation, ammonia and lactate clearance and the presence of danger associated molecular patterns (DAMPS) and inflammation markers (13,35) . Some DAMPS that can be found in the perfusate are aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid, bilirubin and the inflammation markers interleukin 6 (IL-6), and interleukin 10 (IL-10) (13,35).

2.3 Long term machine perfusion

While HOPE and NMP show promising options for reducing IRI and utilizing marginal grafts, there is still a disadvantage of these methods of machine perfusion, being the short perfusion time of max 27 hours (36). HOPE and NMP work fine for reducing IRI but if one wants to potentially repair marginal grafts to ensure better patient outcomes, the perfusion time needs to be extended to several days (13). Recently, Eshmuminov et al. have been able to keep a subset of marginal livers alive and functioning for a week using a newly made normothermic perfusion machine (13). Their liver4life system—as this is what they have called their perfusion machine—was able to solve the major problems encountered when trying to sustain a liver *ex vivo* for a longer period.

In the first place, there is a need for oxygenation and perfusion. NMP and HOPE use either one or two vascular entries, the hepatic artery and the portal vein. When using two entries, mostly only one pump and one oxygenator is used. However, in the body, both the pressure of the blood and the amount of oxygenation differ vastly between these two entries into the liver. To better mimic the body, Eshmuminov et al. gave both entries a different pressure and a different oxygen saturation (both higher for the hepatic artery than for the portal vein) (13). Furthermore, in the body the blood flow in the hepatic artery is not a continuous flow but instead more pulse-like, this is also integrated into the liver4 life system.

The next problem that needed to be solved is proper nutrition in the liver and the ability to have efficient respiration. Normally the liver gets a lot of nutrients from the gut via the portal vein and to mimic this, the nutrients on the liver4life were also added in combination with ursodeoxycholic acid via the portal vein (13). Ursodeoxycholic acid is a secondary bile acid that is added because it is thought to stimulate bile flow and secretion of bile by the cholangiocytes (37). For efficient respiration not only a controlled level of oxygen is needed but also removal of CO_2, so the researchers added a gas exchanger to remove CO_2 from outgoing streams (13).

Another problem related to respiration that was encountered, was the levels of glucose in the liver. Normally the glucose levels in the liver are tightly regulated real-time by both glucagon and insulin. To mimic normal glucose levels, these levels were measured real-time in the perfusate and with the use of an algorithm either glucagon or insulin was added to the perfusate to keep the glucose levels within a healthy range (13).

With efficient respiration also comes a downside and that is the accumulation of waste products. When not properly filtered from the perfusate, these waste products can cause problems

for the liver (13). In the body, the kidneys perform this function and luckily a dialysis membrane can mimic this function of the kidneys *ex vivo*. A similar dialysis membrane is added to the liver4life to filter out waste products from the perfusate (13).

Finally, what normally starts to happen with organs, and also the liver, on a perfusion machine is the death of cells in contact with the machine, this is called local pressure necrosis (13). This is thought to happen because these cells are lying on a surface and are not moving. In the body, the diaphragm is causing constant rhythmic movements of the liver. To mimic this, the liver4life has an inflating and deflating bottom of the machine that mimics the movements of the diaphragm (13).

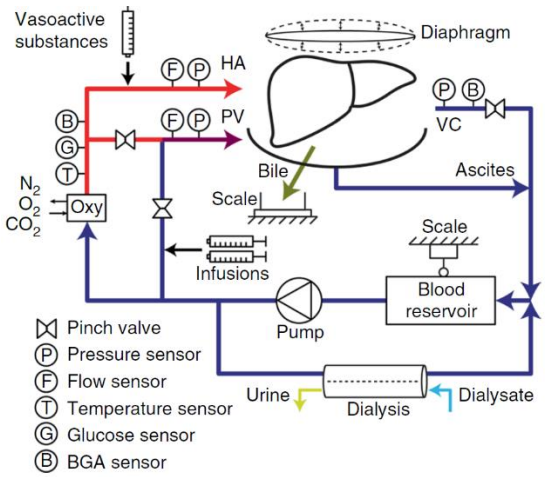

Figure 1: *Schematic representation of Liver4life pump. Oxygen rich blood is shown in red and oxygen poor blood in blue. The system uses one pump but with the use of valves creates different pressures on the hepatic artery and the portal vein. Abbreviations: PV-portal vein, HA-hepatic artery, VC-vena cava, BGA-Blood Gas Analysis. Adapted from (13)*

To summarise the liver4life uses: differentiated oxygenation and pressure, mimicking nutrition and respiratory functionality and simulation of the diaphragm. A schematic representation of the machine is shown in figure 1. This long-term machine perfusion machine has been shown to sustain livers for seven days. The livers were healthy as indicated by several markers; There was metabolic function (shown in lactate and ammonia clearance), there was bile production, and the injury markers ALT and AST were down, together with a decline in inflammation markers IL-10 and IL-6. This machine, with the possibility of longer machine perfusion, may open up possibilities for repairing marginal grafts, regeneration, and liver bioengineering.

3. Genetic modulation and long-term machine perfusion

Over the past years, possibilities to change the genetic expression of cells have increased. Two techniques have been paramount to this progress. The first is the indirect influence on gene expression using RNA interference (RNAi). The second is the direct influence on the genome with the use of CRISPR-Cas9 (38).

3.1 RNAi and the liver

RNAi is a technique that uses small parts of RNA to silence target mRNA. Thereby it hampers the expression of the underlying gene—the target mRNA cannot be translated and thus there is no protein expression of the gene of interest (39). Concerning liver transplantation, there has been a big interest to use RNAi to alleviate IRI by silencing IRI inducing pathways (18). A study in 2017 used RNAi to silence the gene p53 as an alleviation of IRI (40). Another study targeted the gene Fas with RNAi and saw an alleviation of IRI (41). Furthermore, it seems possible to have RNA uptake—necessary for RNAi—in both normothermic and hypothermic machine perfusion (42).

All in all, RNAi seems to have promising effects to alter the liver.

3.2 CRISPR-Cas9 and the liver

The other technique that has recently gotten a lot of attention is CRISPR-Cas9. Pioneered by Doudna and Charpentier—for which they got the Nobel prize in chemistry in 2020—this technique can specifically target and edit genes in the genome (38). With CRISPR-Cas9, the nuclease Cas9 cuts the genome on a desired location in the genome, the cut is precise with the use of a guide RNA that is antisense for the desired place in the genome. After the cut, the gene can be silenced by removing it or another gene can be brought in (38).

Repairing marginal grafts is a promising way to enlarge the pool of available livers. However, there has also been some progress in harvesting grafts from animals for transplantation: xenotransplantation. Recently it was made possible, with the use of CRISPR-Cas9, to humanize organ grafts from pigs by knocking out the (not in humans appearing) gene, α1,3-galactosyltransferase among other genes, causing less immunogenicity. This has for instance been done with a heart which was then transplanted to a baboon, and machine perfusion seemed critical for it to work (15). And recently the first humanized pig kidney was transplanted into a human without any adverse effects in the first 54 hours (after that the brain-dead recipient was taken off artificial ventilation).

However, immunogenicity is not the only concern with pig-to-human transplantation (and xenotransplantation in general), there is also the risk of viruses coded in the genome of the pig to be expressed in the human after transplantation (48). These so-called porcupine endogenous retroviruses (PERV) bring a risk of zoonosis and need to be taken seriously (49,50). Recently however, researchers have created the most advanced pig xenotransplantation model to date with the use of CRISPR-Cas9. In this pig model, researchers deleted three pig genes, added nine human transgenes, and inactivated 25 known PERVs (51). All in all, this shows the power of CRISPR-Cas9 as a tool to be used for transplantation.

3.3 Options for long term machine perfusion

Both CRISPR and RNAi can be powerful tools to help with creating more and better-quality grafts. With machine perfusion it is possible to deliver RNA into the liver for RNAi (42). Interestingly enough, the majority of the work with RNAi and liver grafts has been focused on IRI. However, machine perfusion on itself also protects against IRI as showed in chapter 2. So, the effects of long-term machine perfusion could be more interesting by looking at other genetic pathways to be downregulated. Short-hairpin RNA (shRNA) was used in combination with normothermic machine perfusion to silence MHC expression in both kidney and vascular epithelium (43,44). By doing this the researchers tried to lower the immunogenicity of the graft.

Another study found that senescence can be blocked by using a long non-coding RNA (lncRNA) to suppress a certain part of the LRP130-PGC1a-FOXO4-p21 pathway which plays a big role in senescence (47). One of the things that happens when we age, is the prevalence of more senescent cells in the tissue (45). Furthermore, senescence seems to be one of the causes of age-dependent steatosis (another ECD criterium) in the liver (46). This is another pathway that can be

altered using RNAi in the liver. Lastly, RNAi can also be used to heal a slightly fibrotic liver graft (52). Because you are using the liver on the machine you do not have to worry about systemic effects and can optimize the dose of your therapy for only the liver.

The usage of animals for organ transplantation brings some ethical dilemmas. One is that you have to genetically change an animal while it is alive, possibly hampering its ability to fully function as itself during its life, which can be argued to be of ethical importance for the animal (53). The long-term machine perfusion opens up the possibility to perform the genetic edits necessary for transplantation on the pump instead of in the living animal. This circumvents the need for the creation of a GMO animal and instead, a 'normal' pig liver can be used. However, one must be careful to screen if all the cells in the perfused liver are edited. Otherwise, you still have a chance of unmodified hepatocytes that could still cause problems. At this moment this is still difficult to do.

The major advantage of long-term machine perfusion is that you can monitor the effects of your therapies, CRISPR or RNAi, for a longer time before giving the transplant to the patient. For instance, in repairing steatotic and fibrotic livers, the researchers could immediately see if the therapy works before giving it to the patients. Another thing is that you could monitor whether the genetic edits are performed properly. This can be done by whole genome sequencing of biopsies directly taken from the liver. Furthermore, researchers could monitor markers of the knocked-out genes in the perfusate. This monitoring of the data could give a reason to give a second dose of the therapy until the data confirms the desired outcome. This could even be automated using machine learning as the use of algorithms is already integrated into the liver4life. In the liver4life, the administration of glucagon and insulin to maintain adequate glucose levels is fully automated (13). Overall, there seem to be enough options for using genetic techniques in long-term machine perfusion (Figure 2).

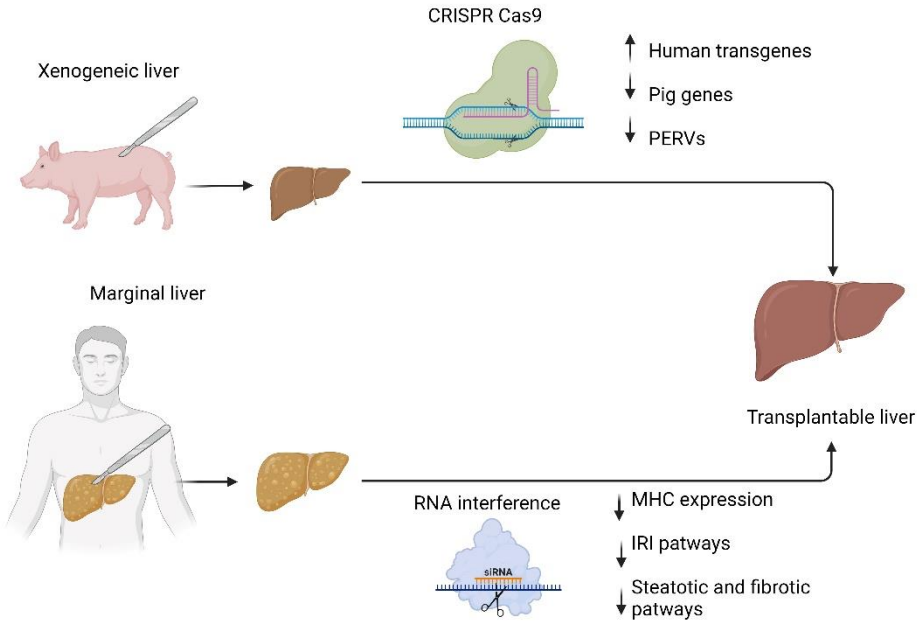

***Figure 2**: Genetic modulation and long-term machine perfusion. Both RNAi and CRISPR-Cas9 can be utilised in long-term machine perfusion to modulate molecular genetic pathways and broaden the pool of available viable liver grafts. Abbreviations: IRI-ischemia reperfusion injury, PERV- porcupine endogenous retrovirus, MHC-major histocompatibility complex. Created with BioRender.com*

4. Regeneration and long-term machine perfusion

4.1 Liver regeneration the basics

The liver is the most regenerative organ in the human body after major injury. This is thought to be of an evolutionary origin because the liver has an enormously high amount of functions—estimated around 500—vital for the body (54). If the liver fails, the body fails. Therefore, the liver can fully recover when as much as 70% of the original liver is taken away in a process called partial hepatectomy (20). There are multiple steps involved in the regeneration of the liver and this is a result of tight changes in gene expression and signal transduction pathways after injury. There is a range of molecular pathways involved in the regeneration, but interestingly not one pathway is solely responsible for liver regeneration, there is a lot of redundancy. This redundancy ties into the evolutionary importance of a functioning liver. During regeneration, there are three steps or phases: 1. Priming/initiation phase, 2. Progression/ maintenance phase 3. Termination phase (55). In all phases, different pathways are important. It is, however, not within the scope of this review to dive into the details of liver regeneration.

4.2 Cells responsible for the regeneration

A big question in the field of liver regeneration is the nature of the cells that account for the great regenerative capacity of the liver. Different from other tissues in the body such as the skin and the gut there does not seem to be single a stem cell population residing in the liver—it seems that there could be three types of cells responsible (56). Firstly, hepatocytes, the major cell population in the liver, could account for the regeneration of the liver (55). The hepatocytes are thought to be able to either repair the liver via fast proliferation or through a phenomenon called hypertrophy, where the cells become bigger to take up more space and mass (20). Secondly, there are some ideas that there may be a subpopulation of oval cells in the liver that could act as a kind of stem cells, but this is highly debated (55). Furthermore, it could also be that both hepatocytes and cholangiocytes (the other major cell type in the liver, responsible for the bile duct) act as stem cells for each other: so-called facultative stem cells (57). And as a last stem cell source, it is hypothesised that cells outside of the liver could differentiate into hepatocytes and in that way repopulate the liver (56).

4.3 Partial hepatectomy, partial transplantation and split liver donors

A lot of clinical information about liver regeneration and regrowth became known after the use of partial hepatectomy. Partial hepatectomy or the utilisation of the regeneration capacity of the liver has been used in a diverse array of clinical applications, this section describes three methods that could be of interest for long-term machine perfusion.

Firstly, hepatectomy and using the regenerative capacity of the liver has been used for the removal of hepatic cancer. There have been multiple techniques to remove the cancerous mass while still keeping a part of the liver alive, this part is then able to recover the full functionality of the liver. A nice overview of the different hepatectomy techniques is given by (19), where they end with the now new popular technique of hepatectomy called Associating Liver Partition and Portal vein Ligation for Staged hepatectomy (ALPPS, also known as ALLPS). In ALPPS, the liver is split *in situ* and subsequently, the right portal vein is ligated. Then in the second operation, the part containing the tumour is taken away (58). The most interesting phenomenon with this report is that the remaining part of the liver can have a 74% increase in volume in an average of 9 days (58).

Another way in which liver regeneration is used, is in partial liver transplantation or living-

donor liver transplantation. Here a living donor gives a part—the left lateral segment (LLS)—of his liver to a recipient (59). That part can regrow to a functional liver in the recipient without serious adverse effects for the living donor (20). This technique has big overlap with another technique that utilises liver ability to regenerate: split liver donation. In split liver donation, a liver graft is split in two parts after procurement (the LLS and the right extended lobe graft) (60). This splitting is similar to the ALPPS approach, although ALPPS is done inside of the body. The split liver approach, although broadening the pool of available grafts, also has some major disadvantages. Firstly, the split liver practice is only preferable with healthy livers (age < 50 years) and not with marginal livers, meaning that at this moment the technique is limited to a subset of the available grafts (60). Second, if you split a good liver—these livers are the best graft, otherwise the splitting will not work—this means that you are sacrificing one good liver for two lesser livers. Ethically this can be justified, you save more people from the waiting list (utilitarianism), but you give them a suboptimal liver. The LLS part of the liver is given to a child, even though the justification for this is that the child has more healthy patient-years in front of him, the ethical situation is far from optimal.

4.4 Possibilities of long-term machine perfusion and liver regeneration

These clinical techniques show us some possibilities of liver regeneration that could be exploited with long term liver perfusion and some problems that can be solved with this technique. The liver can functionally regrow in approximately a week (+/-9 days). And a whole liver graft can be kept alive for one week on a perfusion machine (13). Combining this information opens up a window to regenerate partial livers in the period they can be kept alive on the pump.

4.4.1 Keeping partial livers alive with the help of long-term machine perfusion

To test this hypothesis, the group of the liver4life perfusion machine made some changes to their original setup (14) (Table 2). These changes were done because a partial liver graft is vastly different from a whole liver.

Table 2: *Changes in the liver4life perfusion machine for partial liver grafts.*

Change	Reason
Extra filters	Clogging of the oxygen pumps and other parts because of sectioning debris
Canulations and connecting all bile ducts	Bile leakage, due to a lack of a whole bile duct
Open perfusion system (contact with air)	Lack of a vena cava
Second gas exchanger	Excess oxygen in the perfusate due to a lower gas exchange of the partial liver and due to an open perfusion system

The combination of this long-term machine perfusion technique with partial liver grafts could make it possible to regrow these grafts on the pump. It is possible to keep partial liver grafts viable on the liver4life system for a week without necrosis or apoptosis and the markers for a viable liver were in a normal range (14). In the study by Mueller et al., there was no regeneration seen, however, this was also not the primary outcome of their study. They first wanted to see whether they could keep the partial livers alive on the pump (14). Furthermore, the livers that were used in this study were partial livers that were obtained after surgery for different tumours. Some of these partial liver grafts were influenced by multiple rounds of chemotherapy, making them not the best quality candidates (14).

4.4.2 Options for long-term machine perfusion

Long-term machine perfusion could aid in existing techniques such as partial liver transplantation and split liver transplantation mentioned earlier. The partial liver segment could be connected to a long-term machine perfusion system after procurement. For the split liver for instance both parts could be held on two separate machines. This non-whole liver would then have some time to regain full functional size before transplantation. When combined with life donor transplantation this could help broadening the pool of available grafts.

4.4.3 Aiding regeneration

Because of the limited time on the perfusion machine, it would be nice to enhance the regeneration process. Here the perfusion machine again has a major advantage. Because of its controlled environment, pro-regenerative compounds can be added and monitored in the perfusate (Figure 3). Multiple pro-regenerative pathways in the liver could be stimulated with either drugs or with genetic techniques (RNAi, chapter 3). Because the liver is outside of the body there is no risk of systemic effects by the addition of pro-regenerative compounds, meaning that the most optimal dose could be used without having to worry about other organs.

One molecular pathway that can be used is the hippo-yes-associated protein 1 (hippo-YAP1) pathway. This molecular pathway was found to be crucial for hepatocytes to go into the cell cycle progress (61). There already have been drug screens that have found YAP1 activators (62). Possibly, a YAP1 activator could be added to the perfusate of a long-term perfusion machine.

Another pathway that could be exploited to aid in faster regeneration is the Wnt pathway. Wnt activation also activates hepatocyte proliferation (63). There are already multiple Wnt activators tested for a range of purposes (64). A Wnt activator could also be added to the perfusate.

A whole different thing to look at regarding regeneration is the energy supply that is needed for the regeneration. This energy is thought to come from fat and the process is called transient regeneration associated steatosis (TRAS). ‘Phosphatase and tensin homolog’ (PTEN) downregulation promotes liver regeneration via a TRAS dependent pathway (65). In their study, Kachaylo et al. found that PTEN knockout was able to rescue mice after 91% of their liver was removed; which is lethal in wild-type mice. This shows a huge potential of blocking PTEN, either with drugs or with RNAi. Some compounds can block PTEN function (66). Furthermore, PTEN inhibitors are even shown to have protective effects against IRI making it a double-edged sword when added in the perfusate of a long-term perfusion machine (67).

4.4.4 Unconventional option for long-term machine perfusion

The liver4 life system even opens up the possibility to test more unconventional pro-regenerative pathways. Recently it was found that all cells have a membrane potential and that polarisation of this membrane potential is important for a range of processes in the body including regeneration (68). The membrane potential of the cells is important and can range between -10 and -100 mV. It was found that more proliferative cells (such as cancer cells and stem cells) have a membrane potential that is higher than approximately -36 mV (-36 mV till -10 mV). Less proliferative cells (neurons) have a membrane potential that is lower than approximately -36mV. This gives -36 mV as a threshold between highly proliferative and highly differentiated cells (69). Interestingly the membrane potential of mouse hepatocytes is measured to be -37 mV, just under the proliferative threshold (70).

A potential theory is that the membrane potential of hepatocytes switches to above -36 mV after an injury, causing the hepatocytes to become proliferative and filling up the remaining gap. A possible way in which the membrane potential is changed is by the loss of gap junctions between hepatocytes after injury. This loss of gap junctions in proliferative hepatocytes has been found in rat hepatocytes (71). If this relation with the membrane potential of cells is true, this could be exploited by depolarizing the membrane of hepatocytes by increasing the extracellular K^+ concentration in the

perfusate and thus bringing the liver into a proliferative state (72) (Figure 4). Such modifications in perfusate are only possible in machine perfusion conditions, as K^+ concentrations in plasma cannot be changed in blood without leading to muscle, heart and brain dysfunction. Recently, a subclass of phosphatases was discovered that can hydrolyse phosphoinositides when the membrane is depolarized, voltage-sensitive phosphatases (VSPs) (68). These VSPs consist of a voltage-sensitive domain and an enzymatic domain. The cytoplasmic domain of VSPs has high sequence homology with PTEN (68). Indicating that maybe the PTEN mediate TRAS pathway could be under the influence of changes in the membrane potential. This is all speculative, but the long-term machine perfusion would open up the possibility to real-time measure the membrane potential of the liver cells and alter it if needed.

Overall, by using the regenerative capabilities of the liver and stimulating these on partial liver grafts, long-term machine perfusion would broaden and enhance the pool of available donors.

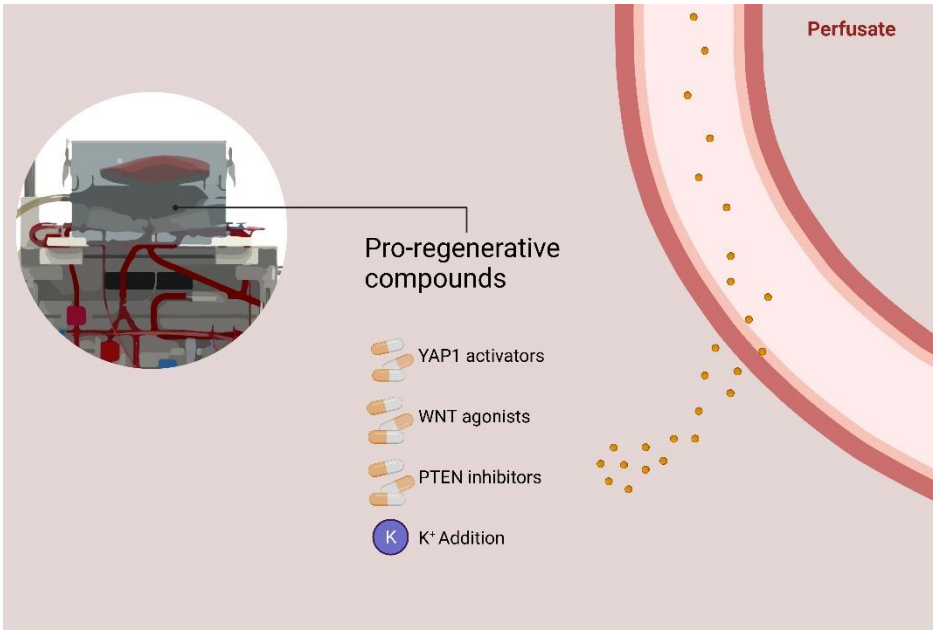

***Figure 3**: pro-regenerative compounds added to the perfusate. Because of the ex-vivo nature of long-term machine perfusion, drugs and other treatments to enhance regeneration can easily be added to the perfusate. Abbreviations: YAP1- yes-associated protein 1, PTEN- Phosphatase and tensin homolog. Created with BioRender.com*

5. Bioengineering and long-term machine perfusion

Instead of just looking at organs, researchers have also looked at other ways to overcome organ shortages and shorten the waiting lists. One of these ways is bioengineering (22). This chapter will highlight two techniques that are being investigated and are different from transplantation—cell-based techniques and bioengineering using scaffolds.

5.1 Cell-based techniques

Cell-based transplantation has been around since the 1990s and is approved for use in the clinic (21). In this technique, healthy hepatocytes are transplanted into diseased livers of patients. These hepatocytes then populate the diseased liver and are thought to give relief. The advantage of this technique is that it is less invasive for the recipient than normal liver transplantation. Instead of a risky operation where the recipients diseased liver needs to be removed, here only a small portion of cells is given to the patient (21). Furthermore, because of the low risk, another subsequent procedure can be performed if required. Lastly, in theory, the patient's autologous cells could be used, if these cells were for instance fixed from a genetic problem in vitro (17).

There are however, also major disadvantages to this technique (21,23). To have an effect, a large number of hepatocytes is needed. Primary hepatocytes are difficult to maintain in vitro and lose their functionality (73). Furthermore, the main source of hepatocytes for transplantation are livers rejected for liver transplantation (21). Most of the time these livers are not of the best quality, meaning that the cells are also not of a good quality (marginal liver, which probably checks all the extended criteria to be discarded). Liver perfusion could be a better usage of these discarded livers. Another disadvantage is that it is difficult to deliver a large number of cells to the liver in a living patient. Because of safety measures a maximum of 2 X 10^8 cells per kg of bodyweight can be safely infused, and this cell dose cannot even be delivered in one single infusion (21). Lastly, the cells need an advantage over autologous cells already present in the liver of the patient to have an effect. This can be induced with invasive techniques (irradiation, partial hepatectomy, ALPPS) but it is suboptimal (21). Furthermore, the grafting efficiency of the hepatocyte transplantation is low, between 0,1% till 0,3% (74). This means that a large portion of the injected cells die, leading to the formation of damage signals and inflammation signals in the body, which could hamper the function of the remaining healthy liver (75).

Other cells have also been investigated to overcome the major disadvantages of using primary hepatocytes. Mesenchymal stem cells have been reported to be able to transduce into hepatocyte-like cells (76). Another more promising technique is looking at pluripotent cells. These include embryonic stem cells and induced pluripotent stem cells (iPS). It is possible to make hepatocyte-like cells from iPS (77). However, embryonic stem cells have some ethical issues and iPS have a risk of becoming teratogenic (78). Another way is to try to keep the hepatocytes in a 3D environment, this is thought to help the hepatocytes to keep their function (23,73).

5.1.1 Organoids as a breakthrough in cell-based techniques

Recently there has been a breakthrough in the creation of 3D cell culture models, the so-called organoid (79). The organoid is described as: '3D multicellular *in vitro* tissue construct that mimics its corresponding *in vivo* organ, such that it can be used to study aspects of that organ in the tissue culture dish' (79). Organoids have multiple advantages: They can proliferate in a way that even one stem cell could give rise to a million cells within two months (73). They are stable, hepatocyte organoids can be kept for at least 8 months and can be freeze-thawed multiple times (23,81). There

are even bipotent organoids that can differentiate into both hepatocyte-like cells and cholangiocyte-like cells (82). Organoids can be cultured from small liver biopsies (82). Hypothetically, a small part of a patient's liver can be taken that is not completely diseased and that part could be grown up to a viable organoid *in vitro*, possibly these could then be edited with gene editing techniques described in chapter 3.

It was recently found to be possible to expand hepatocyte organoids and these could be transplanted into mice and successfully repopulate the injured host liver (80,81) However, one must take into account that the engraftment in these studies is still a fairly modest and only seen in a mice model. Concerning machine perfusion, cholangiocyte organoids have recently been transplanted into a human liver (24). These organoids were made from biliary epithelial cells and *ex vivo* normothermic perfusion was used to deliver the organoids to the region of the biliary tree. This study showed that it was possible to repair the biliary tree with these organoids and serves as a proof-of-concept study that machine perfusion could aid in this regard. This is an especially interesting study because the repair of the biliary tree has been shown, which is one of the most fragile parts of the liver in the transplantation process.

5.2 Bioengineering using bioscaffolds

Whereas the previous bioengineering techniques still used the liver or at least the cells from the liver as a starting point and a target for repair, this section describes some new research that is stepping away from this focus on an existing liver and is instead trying to make a liver by populating a bioscaffold with cells.

In decellularization, cells are removed from the existing organ. In that way, only the extracellular matrix remains (25,83). There are two ways for decellularization, chemical and enzymatic. An organ is thought to be fully decellularized when the extracellular matrix does not contain any cells (83). This can be tested by looking if there is DNA present and by for instance DAPI staining. When the organ is fully decellularized, the ECM can be repopulated with single healthy cells (hepatocytes) and organoids. Verstegen et al. performed the first decellularization on a whole human liver. The decellularized liver remained in its 3D shape including vascularisation and bile ducts (25). Furthermore, the decellularized organ could be repopulated with hepatocytes and these healthy hepatocytes found their place where they normally reside in the liver. Although there is no clinical transplantation performed with a human recellularized liver, a repopulated liver has been transplanted into a rat giving proof of principle (84). One of the biggest disadvantages of recellularization is that you need a large number of cells that need to be cultured *in vitro*. Also, the liver does not consist only of hepatocytes but also a plethora of other cells that in some way also need to be repopulated into the empty ECM scaffold.

5.2.1 Bio scaffolds from scratch

Instead of attempting to make a liver from a decellularized whole liver, a liver could be made from scratch by using bioscaffolds . With bioscaffolds, a natural or synthetic scaffold is made where cells can grow to form a kind of artificial organ, for instance, a liver. This scaffold must mimic the extracellular matrix for the cells to have their proper function. At this moment hydrogels are used for this (85). A hydrogel contains natural or synthetic hydrophilic polymers to form a network that has a flexibility that is similar to natural tissues (85). These hydrogels can be natural hydrogels, which are made from natural products. These have the advantage to mimic the body better and are better when the artificial liver would be transplanted inside the body (85). Recently a decellularized liver was shown to be able to form a natural hydrogel (86). However, one could argue that it is somewhat pointless to decellularize a whole liver with all its vasculature and ducts. You destroy this architecture to make a hydrogel, which can then be used to make a liver from scratch, where you again would need that architecture. Another option is to make synthetic hydrogels but these are not natural,

possibly giving some problems when transplanting to a recipient and they may not encapsulate all the properties of the ECM (85).

One advantage of hydrogels is that they can be used for bioprinting. This makes it possible to print precisely the kind of tissue/scaffold needed, and there are a lot of bioprinting techniques available now (87). The disadvantage of bioscaffolds is that when made from natural hydrogels, there are mechanical weaknesses, batch-to-batch differences and the bioscaffolds could be animal-derived which poses some ethical issues (85). Furthermore, the main natural hydrogel now used, Matrigel, has the disadvantage of coming from a xenogeneic tumorigenic source, making it difficult to get clinical approval (22). Above that, the main disadvantage of both natural and synthetic hydrogels is that it is difficult to achieve proper zonation (the metabolic gradient of hepatocytes) in *in vitro* systems (88). Additionally, it is still not possible to obtain proper vascularization of the bioscaffold (22,87). Without vascularisation of the tissue no oxygen and nutrients can reach the cells, leading to hypoxia and apoptosis (22).

5.3 Best of both worlds, combining bioengineering and long-term machine perfusion

Cells given in cell-based techniques must find a place where they can populate. In cell transplantation, this is the liver of the patient. In the other techniques, this is an ECM scaffold. When a liver is on the pump with long-term machine perfusion, the cells could use that liver as a scaffold (Figure 4). The cells could easily be given by adding them to the perfusate and would enter the liver either via the portal vein or via the hepatic artery. Another advantage is that the engraftment can be monitored *ex vivo* and a potential need for a second dose could be evaluated. It would even be possible to give autologous cells to a liver on the pump. The most healthy cells from the perfused liver could be harvested, then grown *in vitro* and be gene-edited and then be placed back in the system (17).

Organoids are a great technique to expand and maintain a large number of cells in a 3D environment. However, on themselves, the organoids lack the key architectural functions (vascularization, immune cells) to allow them to be effective as a substitute for liver transplantation (22). With the help of long-term machine perfusion organoids could be given *ex vivo* to a graft to help repair some diseased parts of the graft. IRI is mostly present in the bile ducts after ischemia (chapter 2). It is possible to give cholangiocyte organoids to the graft *ex vivo* on a normothermic perfusion machine (24). Here, long-term machine perfusion has the advantage that there is time to evaluate the success of the engraftment of your organoids and you can directly evaluate other markers of success of your engraftment such as biliary leakage.

Another thing that is explored with bioengineering is to mimic all the functions of the liver outside of the body. This is called a bioartificial liver (89). The main disadvantage with bioartificial livers is that not all liver functions can currently be replicated (22). If it would be possible to keep a liver alive for longer stretches (weeks, months, years) on a perfusion machine, it could be possible to use long-term perfusion as a kind of bioartificial liver. However, this option is still far away, and transplantation is always more beneficial because it gives freedom of movement to the patient.

The biggest problems with bioscaffolds from scratch—zonation and vascularization—could be overcome with long-term machine perfusion. When you are working with a perfused liver as a bioscaffold, you already have the ECM architecture for proper zonation of the hepatocytes, and there is also already a vascular network for the proper vascularization.

Finally, the technique of decellularization does not seem appealing for the long-term machine perfusion as the graft on its own can already work as a scaffold and decellularization would be a waste of hepatocytes. However, one could hypothesize about partial decellularization of a liver graft on the long-term perfusion machine. Here one could use a kind of split liver experimental setup: splitting the liver on the machine and keeping one part alive with the use of the machine. Then the

other part is decellularized. This decellularized part could then be repopulated with healthy hepatocytes, possibly even organoids from autologous cells of the potential recipient. Then the two parts could be ligated to form a whole liver again. What also could be done, is to only decellularize the part of the liver with a lot of steatosis, and then repopulate with healthy hepatocytes.

All in all, there are a lot of exciting new techniques that try to overcome the need for whole liver graft transplantation. Because these techniques all have some drawbacks, long-term machine perfusion could work as a way to bring these techniques to patient.

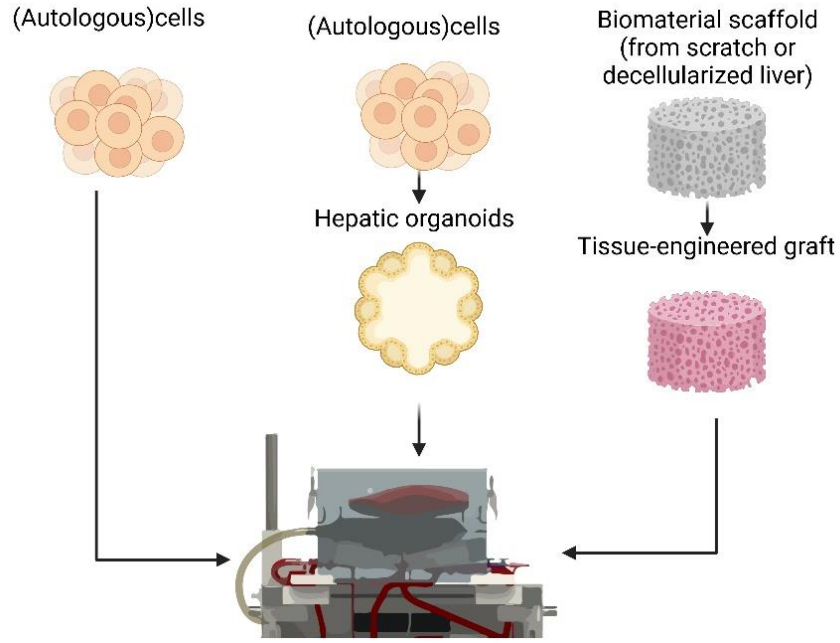

Figure 4: *Bioengineering and long-term machine perfusion. Long-term machine perfusion can be used in three ways. As a scaffold to give autologous cells, as a scaffold to for hepatic organoids, and as a scaffold for decellularization and repopulation. Created with BioRender.com*

6. Discussion and conclusion

At this moment research into machine perfusion is a hot topic. The number of publications per year about machine perfusion has seen a sharp increase over the last couple of years (Figure 5).
This review has shown why long-term machine perfusion can be seen as one of the most exciting new developments in machine perfusion (Figure 6).

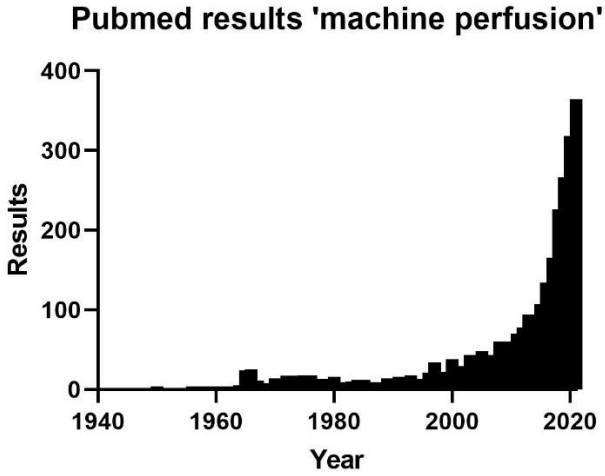

Figure 5: *Publications mentioning 'machine perfusion' per year. Source: pubmed.gov*

As seen in chapter 2, machine perfusion on itself is a major development in organ preservation and can protect against damage induced by ischemia. Furthermore, machine perfusion opens up the ability to monitor the quality of liver grafts *ex vivo*. By mimicking the necessary bodily functions to keep a liver graft alive on the pump, the new development of long-term machine perfusion adds extra benefits to the existing benefits of machine perfusion.

The subsequent chapters highlighted the potential that long-term machine perfusion could bring for liver graft enhancement. When combined with novel genetic modulation techniques such as RNAi and CRISPR-Cas9, long-term machine perfusion could enhance graft quality and improve immunogenicity. The *ex vivo* characteristics of long-term machine perfusion eliminate some dilemmas with gene editing such as the ethical dilemma of gene editing a (human) being. Furthermore, the long period of perfusion enables rigid whole-genome sequencing to screen for possible off-target effects of the genetic modulation, making the technique safer.

Because of the liver's great regenerative capacity and its ability to regenerate to functional capacity in a mere couple of days, long-term machine perfusion also has some major benefits. Partial liver grafts can be kept alive with the use of machine perfusion for a longer period. Furthermore, the nature of the machine enables the addition of pro-regenerative compounds to the perfusate. Because of its *ex vivo* nature, more experimental approaches such a changing the membrane potential could be explored. This makes long-term machine perfusion a promising platform to investigate and utilise *ex vivo* regeneration of partial liver grafts.

Some recent research to overcome the shortages of liver transplants has been focused away from actual transplants and more onto other techniques such as cell therapies and bioengineering. Long-term machine perfusion has the potential to work as a way to combine these techniques and clinical liver transplantation, acting as an advanced bioscaffold.

Interestingly enough, some bioengineering techniques could maybe be combined with the

regeneration of partial liver grafts. Instead of solely relying on the partial liver's cells, one could hypothetically add single hepatocytes or hepatocyte organoids to the partial liver graft to provide more cells to the partial liver grafts. Hepatocyte and cholangiocyte organoids can be made from small biopsies. To overcome immune rejection, possibly a biopsy from the partial liver graft on the pump could be taken. From the biopsy, hepatocyte and cholangiocyte organoids could be made *in vitro* and given to the partial liver graft. The genetic modulation techniques can also be exploited for regeneration of partial liver using long-term machine perfusion. Several molecular pathways are important for regeneration, and these could be stimulated *ex vivo* for regeneration.

Although long-term machine perfusion opens up some major possibilities and has great advantages, there are also some disadvantages of this technique that need to be discussed. Firstly, the liver4life is vastly more complex than conventional perfusion machines. With all its extra functions and the extra cost of personnel to learn to work with the machine, the costs are higher than conventional machine perfusion and considerably more expensive than normal SCS. However, machine perfusion, in general, protects against IRI which could significantly lower the cases of biliary problems for recipients. As biliary problems are one of the major causes for retransplantation, this would decrease the cost and create more available grafts. Furthermore, long-term machine perfusion could enable the use of grafts that would otherwise be discarded, leaving fewer patients on the waiting lists. Take into account that the annual healthcare cost for an end stage-liver patient is also high (+/-$60.000/year) (90). The liver4life is the first prototype of a long-term perfusion machine, and as seen with all big developments in technology, eventually the costs will go down if the technique gets some attention.

What needs to be considered is that the technique of long-term machine perfusion as proposed by Eshmuminov et al. is novel and no actual regeneration of partial liver grafts has been shown yet. Therefore, most of the advantages proposed here in this article are hypothetical advantages. However, some work on other perfusion machines such the cholangiocyte organoid transplantation and RNAi to tackle IRI, show that research is being done on perfusion machines and that this research is promising.

Another thing that needs to be considered is the consent given by organ donors. At this moment an organ donor gives consent for giving an organ after passing. This is consent for donating an organ, not for repair and enhancement of the organ, with or without genetic techniques. This 'tinkering' with their organ before transplantation could be a disconcerting thought for some donors. In the Netherlands (and also other countries in the world) the government works with an opt-out system. This means that a person is a donor *unless* he indicates that he does not want to be one. A discussion needs to be raised into whether the choice of a donor needs to be considered regarding the repair and enhancement of the donated graft.

As of this moment, the liver is the only organ that can be kept alive for a longer period. For future research, the liver4life approach (mimicking the body as much as possible) could be investigated for other organs such as the kidneys and lungs. Following logically from the lack of actual data, research with the liver4life system into regeneration could be performed in the future, also by looking at the addition of regenerative compounds. The period of seven days that was achieved by Eshmuminov et al. was chosen arbitrarily, so possibly this could be extended further. An experiment where the longest possible duration is tested could be performed. In addition, because of its *ex vivo* character, also new developments surrounding regeneration and development could be investigated, such as the role of epigenetics and bioelectricity. Finally, at this moment only one research group is working on the liver4life machine, and regarding open science, this technique could be shared with other transplant centres around the world.

Long-term machine perfusion opens up new ways to help more people on the waiting lists for liver transplantation. There still needs to be more research into this area for it to become a viable

clinical solution, but the same was the case with liver transplantation in its infancy. In this developmental phase, it is wise to listen to the words of the founder of clinical liver transplantation, Thomas Starzl: “What was inconceivable yesterday, barely achievable today, often becomes routine tomorrow''.

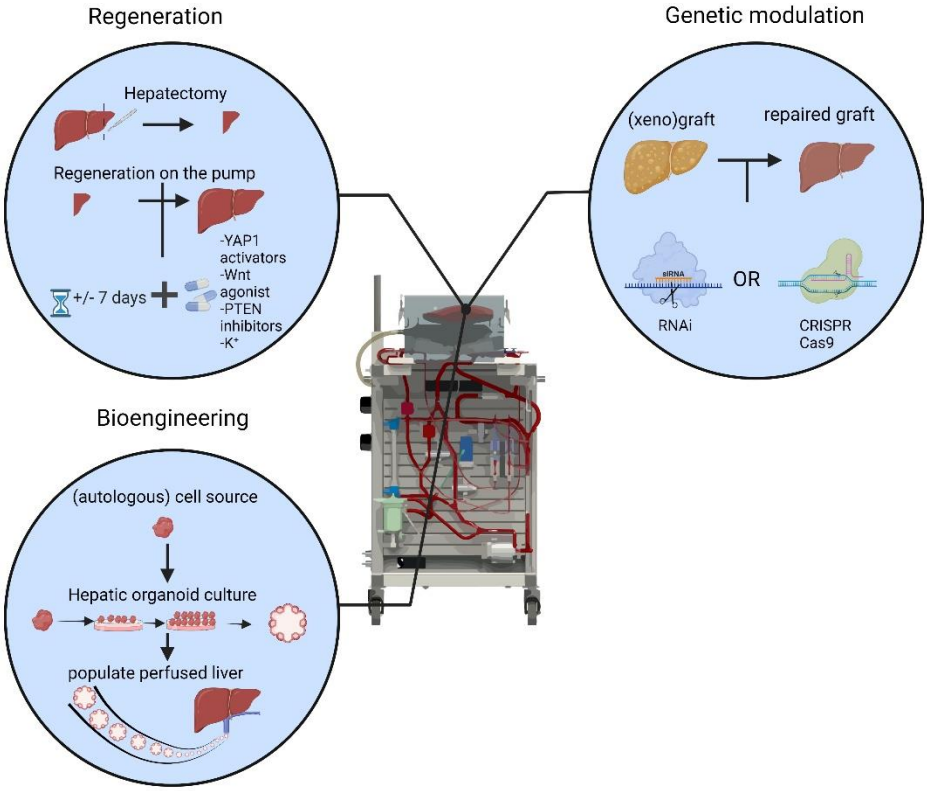

***Figure 6**: Graphical summary. Long-term machine perfusion opens up possibility to combine exciting new techniques with a transplantable graft. Created with BioRender.com*

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