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REVIEW

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Contents

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REVIEW

Therapeutics administered during *ex vivo* liver machine perfusion: An overview

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Abstract

Although the use of extended criteria donors has increased the pool of available livers for transplant, it has also introduced the need to develop improved methods of protection against ischemia-reperfusion injury (IRI), as these "marginal" organs are particularly vulnerable to IRI during the process of procurement, preservation, surgery, and post-transplantation. In this review, we explore the current basic science research investigating therapeutics administered during *ex vivo* liver machine perfusion aimed at mitigating the effects of IRI in the liver transplantation process. These various categories of therapeutics are utilized during the perfusion process and include invoking the RNA interference pathway, utilizing defatting cocktails, and administering classes of agents such as vasodilators, anti-inflammatory drugs, human liver stem cell-derived extracellular vesicles, and δ-opioid agonists in order to reduce the damage of IRI. *Ex vivo* machine perfusion is an attractive alternative to static cold storage due to its ability to continuously perfuse the organ, effectively deliver substrates and oxygen required for cellular metabolism, therapeutically administer pharmacological or cytoprotective agents, and continuously monitor organ viability during perfusion. The use of administered therapeutics during machine liver perfusion has demonstrated promising results in basic science studies. While novel therapeutic approaches to combat IRI are being developed through basic science research, their use in clinical medicine and treatment in patients for liver transplantation has yet to be explored.

Key Words: Therapeutics; Liver transplantation; *Ex vivo* machine perfusion; Ischemia reperfusion injury; Organ preservation; Extended criteria donors

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Core tip: The use of extended criteria donors has increased the donor pool of available livers for transplant but has also introduced other hurdles in protecting these vulnerable organs against ischemia-reperfusion injury (IRI). Current basic science research is aimed at mitigating the effects of IRI during the transplantation process by administering therapeutics during *ex vivo* liver machine perfusion. Of interest include therapeutics aimed at invoking the RNA interference pathway, utilizing defatting cocktails, and administering classes of agents such as vasodilators and anti-inflammatory drugs to reduce the damage of IRI following liver procurement and transplantation for ultimate preservation of the organ.

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INTRODUCTION

The overall increasing success of liver transplantation over the last several years has unfortunately introduced one of the most significant hurdles to date - longer waiting lists and increased mortality while on the waiting list. In an effort to combat the organ shortage, transplant centers have extended the criteria for donors often considered for transplantation. Common categories of extended criteria donors (ECDs) now being included in the context of the donor liver pool include donation after cardiac death (DCD), hepatic steatosis, donors of advanced age, organs that have experienced prolonged normothermic and cold storage, and donors with an increased infectious risk. The inclusion of ECD in the donor pool has increased access to previously deemed un-transplantable organs by 77% while reducing the mortality of those on the waitlist by over 50%[1].

While inclusion of ECDs has positively impacted the pool of livers available for transplant, the new criteria has also highlighted the need for improved methods to ameliorate ischemia-reperfusion injury (IRI) in these less than optimal organs due to a weakened defense against ischemia-reperfusion injury during the transplantation process[2]. Ischemia-reperfusion injury occurs when blood supply to an organ is inhibited and then later restored, with this process resulting in oxidative damage, cell death, and generation of reactive oxygen species (ROS)[3]. The hepatic molecular pathways involved in IRI are complex with liver sinusoidal endothelial cells and hepatocytes as the initial targets for cell death as a result of ATP depletion. Neutrophils and macrophages then accumulate in the liver leading to ROS generation while hepatic stellate cells then become activated to aid in recovery, ultimately leading to fibrosis of the allograft[4-6].

Targeting specific candidates implicated in hepatic IRI therefore becomes challenging due to the complex molecular pathways that become activated. Some of the activated pathways and molecules include the complement cascade, the innate immune response and toll-like receptors (TLRs), CD4 T lymphocytes, inflammatory cytokines propagating the post-inflammatory response, nuclear factor κB (NF-κB) leading to production of TNF-α, adhesion molecules, apoptotic pathway activation, and ROS production and release[7,8]. As it will be discussed, basic science research focused on hepatic IRI has attempted to target many key mediators implicated in the IRI cascade. Most studies rely on using a combination of therapies that block multiple, perhaps redundant, reperfusion injury pathways in order to achieve a significant reduction in injury and overall improvement in graft function[9].

There currently exists no established clinical therapies to avoid IRI, and the main method implemented to reduce IRI relies on limiting the cold preservation period and re-warming of the organ[2]. Other methods of graft protection prior to transplantation include immunosuppressive agents and modulation of the immune response. Immunosuppressive therapies, particularly in the setting of kidney transplantation, has proven to be advantageous when the donor is treated prior to graft procurement or when the graft is directly treated during perfusion or cold storage[10].

While static cold storage (SCS) remains the gold standard for liver preservation, *ex vivo* machine perfusion (MP) preservation of the liver is gaining attention from both

basic scientists and transplant surgeons alike. *Ex vivo* MP is an attractive alternative to SCS due to its ability to continuously perfuse the organ microcirculation, effectively delivery substrates and oxygen required for cellular metabolism, therapeutically administer pharmacological or cytoprotective agents, and continuously monitor organ viability during perfusion[2].

CURRENT *EX VIVO* LIVER MP CLINICAL TRIALS

Several recent liver transplantation clinical studies demonstrate the logistical feasibility and safety of MP in the hospital setting. Ravikumar *et al*[11] published the first Phase 1 normothermic perfusion trial of 20 patients who underwent NMP liver transplantations demonstrating decreased AST levels compared with controls during the first 7 d and a 95% one-year patient survival rate in the NMP group. Although there was no statistical difference in the primary outcome, this study reported the first use of NMP as logistically feasible and safe for use in the clinic[11]. Selzner *et al*[12] report the use of normothermic *ex vivo* liver perfusion in 10 human liver grafts using an albumin-based Steen solution with comparable outcomes to traditional SCS post-liver transplantation. In addition, Czigany *et al*[13] report an ongoing open-label, phase 2 randomized controlled trial using HMP in liver transplantation from ECDs although primary and secondary endpoints and extent of IRI have not yet been published.

Another recent advancement in human liver MP involved the first randomized controlled trial of 220 liver transplantations performed by Nasralla *et al*[14] at normothermic preservation conditions and demonstrated a 50% lower level of graft damage compared to the traditional cold static method of preservation in addition to 50% fewer discarded organs in the normothermic machine perfused group. This trial describes the novel expansion of normothermic human liver MP from experimental bench studies to introduction into clinical practice and demonstrates its benefit over the traditional cold method of preservation. While a larger study is needed to determine the impact of NMP on liver graft and patient survival, preliminary results indicate an exciting future for normothermic liver preservation.

Although the purpose of this review is not to highlight every current clinical trial involving *ex vivo* liver MP to date, it is worth noting that the number of both prospective and retrospective studies investigating the role of HMP *vs* SCS in human liver transplantation is increasing but still remains limited. These studies have been conducted in a variety of countries including the United States, Switzerland, the Netherlands, and the United Kingdom[15]. The clinical use of *ex vivo* liver MP may be viewed as a limitation due to it being in its infancy as a standard therapy in liver transplantation. However, the basic science advances that will be highlighted in this review continue to expand the applications of *ex vivo* liver MP closer to its acceptance as a more reliable, efficacious method of organ preservation for liver transplantation.

BASIC SCIENCE *EX VIVO* LIVER MP THERAPEUTICS

In this paper, we review basic science advances made in the area of therapeutics administered specifically during *ex vivo* liver machine preservation transplantation models (Table 1) and their importance in extending the donor criteria for liver transplantation in a clinical setting. While therapeutics for this review were only considered in the context of the liver, administered therapeutics during MP of additional organs such as the lungs, heart, and kidneys are currently being explored in an effort to reduce IRI during transplantation and increase the available organs suitable for transplantation. Therefore, the impact of therapeutics administered during MP to mitigate IRI during and post-organ transplantation holds tremendous potential to increase the donor pools of many transplantable organs while simultaneously reducing the waiting time for those hoping to gain a second chance at life.

RNA interference and its therapeutic role in the liver during ex vivo MP

One of the most recent basic science advances in liver MP therapeutics includes utilization of the RNA interference (RNAi) pathway to silence specific genes implicated in IRI. RNAi selectively silences genes by the RNA-induced silencing complex (RISC) upon hybridization with the target mRNA, subsequently leading to degradation of the mRNA by Argonaute, an RNase H enzyme. If there are mismatches between the RNA complex and the target mRNA, silencing can occur at the post-

Table 1 Major categories of *ex vivo* machine perfusion therapeutics in liver transplantation

Category	Agents
RNAi pathway	siRNA
	Anti-sense oligonucleotide (Miravirsen)
Defatting cocktail	Variable
Vasodilators	Prostacyclin
	BQ123 and Verapamil
	Prostaglandin E1
Others	Anti-inflammatory agents
	Human liver stem cells extracellular vesicles
	δ-opioid agonist (Enkephalin)
	NLRP3 inflammasome inhibitor mcc950

RNAi: Ribonucleic acid interference; siRNA: Small interfering ribonucleic acid; NLRP3: Nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3.

transcriptional level leading to translational repression or exonucleolytic degradation[16].

The overall RNAi mechanism contains several unique regulatory RNA molecules including microRNA (miRNA), small interfering RNA (siRNA), and short-hairpin RNA (shRNA). There have been only a handful of reports of the RNAi pathway being implemented in the context of animal liver transplantation. For example, Li *et al*[17] employed a rat liver transplantation model to study the effects of Fas siRNA on IRI. Hydrodynamic injection of 200 nmol/kg Fas siRNA transfection of the penile vein was performed 48 h before liver procurement. Measurements from the recipient rats demonstrated reduced ALT levels, decreased apoptotic index levels, and reduced Fas mRNA and protein levels 24 h after blood reperfusion.

Contreras *et al*[18] delivered caspase-8 or caspase-3 siRNA in an *in vivo* C57BL/6 mouse model *via* the portal vein by high-volume injection 1 hour before induction of ischemia for 90 min. Results demonstrated a reduction in caspase-8 and caspase-3 gene expression of greater than 60% following siRNA injection. In addition, siRNA-treated mice showed improved survival for greater than 30 d when treated with caspase-8 siRNA (30%) and caspase-3 siRNA (50%) compared to controls where all of the mice died within five days after being subjected to total liver ischemia.

In addition, Wu *et al*[19] targeted interleukin-1 receptor-associated kinase-4 using shRNA (IRAK-4-shRNA) in a rat liver transplantation model to prevent IRI. IRAK-4 is implicated in the downstream signaling pathways of lipopolysaccharide (LPS) activation of IRI in addition to its role in Toll-like receptor (TLR) and IL-1R mediated innate immune responses and was therefore selected as an ideal candidate for shRNA targeting to prevent IRI[19-23]. In this study, rat liver grafts were perfused *via* the portal vein with a plasmid expressing IRAK-4-shRNA for 4 min during the cold ischemia time and then stored in University of Wisconsin (UW) perfusion solution for a period of 6 h prior to transplantation. Post-liver transplantation results indicated improved liver function, preserved tissue architecture, decreased IRAK-4 mRNA and protein levels, decreased NF-kB, TNFa, IL-6, and IL-1B levels over a period of 180 minutes post-reperfusion[19].

While these previous studies demonstrated the utilization of the RNAi pathway to selectively target genes implicated in IRI by introducing siRNA or shRNA using hydrodynamic injection, our group most recently reported the first use of *ex vivo* liver MP as a method of siRNA delivery prior to rat liver transplantation[24]. In this study, siRNA targeting the Fas receptor was added directly to the perfusion solution and MP of the liver was maintained at either hypothermic (4 °C) or normothermic (37 °C) conditions using a closed loop perfusion circuit[24]. The Fas siRNA construct was conjugated to invivofectamine lipid nanoparticles and the siRNA-lipid complexes were perfused *via* the portal vein for 4 h. Confocal imaging studies revealed Fas siRNA distribution throughout the liver sinusoids and central veins in both the hypothermic and normothermic conditions[24].

This study demonstrated for the first time siRNA uptake and distribution in the

liver following *ex vivo* MP at hypothermic and normothermic conditions. While future studies will examine the effects of siRNA uptake and delivery using MP in a rat transplant model, this report highlights the exciting use of RNAi in the context of *ex vivo* liver preservation.

In addition, our group utilized a similar *ex vivo* normothermic machine preservation system as aforementioned to silence the p53 tumor suppressor gene in a rat liver damage model. Rats were injected with p53 siRNA conjugated to invivofectamine prior to initiation of liver damage[25]. To induce liver damage, the liver hilum was clamped for 15 minutes. Confocal microscopy studies revealed p53 siRNA uptake into the machine perfused liver with reduced levels of the inflammatory cytokines IL-1, IL-6, and TNFα compared with controls[25].

The utilization of the RNAi pathway in the context of machine preservation of the liver is a new concept with promising therapeutic value. Although administering therapeutic siRNA to human donors before procurement is clinically feasible, the cost of therapy and potential for side effects increase[26]. The therapeutic dosage of siRNA delivery to the donor liver could be efficiently delivered with little to no side effects to other organs as the siRNA therapeutic concentration would be based upon the weight of the donor liver rather than the weight of the whole donor at the time of procurement[26]. In addition, preliminary studies demonstrate the uptake of siRNA in both hypothermic and normothermic temperatures in a rat model suggesting that perfusion temperatures have little to no effect on uptake while future studies will be aimed at addressing the effect of temperature on siRNA efficacy in silencing its target in a transplant model. Therefore, inclusion of siRNA in the MP solution for liver transplant models demonstrates promising and exciting results while also holding tremendous therapeutic value for future use in the operating room.

It is also worth noting that anti-sense oligonucleotide (ASO) therapies have also recently been used against hepatitis C virus (HCV) infection. Mir-122 is a known miRNA of the liver and is a target of miravirsen, which is currently in Phase 2 clinical trials for treatment of HCV[27]. Miravirsen has been shown to nearly completely eradicate HCV presence in cell culture. In addition, miravirsen was shown to sequester miR-122 (a necessary agent for HCV infection) in pig liver without inducing harmful effects[28]. Miravirsen is a recent exciting advancement in liver-targeted therapies as it could significantly increase the donor pool by allowing the transplantation of HCV^+ livers.

Although the concept of gene silencing using RNAi in liver transplantation MP models is in its earliest stages of development and optimization, it holds exciting potential as a future therapeutic to combat the damage caused by IRI as a result of liver transplantation.

Defatting cocktails to reduce steatosis during MP of the liver

The increasing prevalence of obesity and metabolic syndrome defined as insulin resistance, hyperlipidemia, hypertension, and hyperglycemia, have contributed to potential donors developing hepatic steatosis. Hepatic steatosis is defined as having intrahepatic triacylglycerol (TAG) of at least 5% of the total liver weight or 5% of hepatocytes containing lipid vacuoles without a patient history of secondary contributing factors including viral infection, excess alcohol intake, or drug treatments[29]. Reports indicate that 33% of the United States adult population has nonalcoholic hepatic steatosis[30].

Potential donors with hepatic steatosis are now included in the ECD donor pool, however this criterion has implications in post-transplant outcomes. An analysis of the Scientific Registry of Transplant Recipients reported that liver allografts with greater than 30% macrovesicular steatosis were independently predictive of reduced 1-year graft survival[31].

Furthermore, steatotic livers are particularly susceptible to IRI, increasing the risk of postoperative morbidity and mortality after liver surgeries and liver transplantations[32]. Recent basic science evidence also provides support that steatosis exacerbates the effects of IRI. Chu *et al*[33] demonstrated in a rat model of liver steatosis that the steatotic-IRI livers had elevated ALT levels, evidence of histological injury, and impaired mitochondrial complex-1 function after partial hepatic normothermic ischemia compared to the lean liver controls. Liss *et al*[34] provided evidence in a murine model of hepatic IRI that steatosis increases plasma ALT, inflammatory cytokine levels such as TNF-α and IL-6, and necroptosis markers RIPK1, RIPK3, and MLKL compared with low-fat diet controls.

Gehrau *et al*[35] explored the effect of IRI on immune response pathways in human graft biopsies classified based upon the degree of graft steatosis. The results showed that compared with non-steatotic control grafts, the steatotic grafts had significant

post-transplant innate immune response activation of IL-6, IL-8, and IL-10, macrophage production of nitric oxide (NO) and ROS, and neutrophil and leukocyte recruitment around the sites of hepatocyte lipid accumulation[35]. Ramachandran *et al*[36] also demonstrated that NFκB P65 is associated with the inflammatory pathway implicated in IRI and necrosis in rat steatotic liver transplantation. Multidrug donor preconditioning of steatotic rat liver grafts has also been reported to abolish the IRI inflammatory mechanism while preventing an increase in parenchymal cell death following cold storage and reperfusion[37].

A systematic review examining the animal model experimental studies investigating hepatic steatosis and IRI found that livers with > 30% macrovesicular steatosis were associated with a lower graft and recipient survival rate as a result of the effects of IRI[38].

More recently, liver MP has been investigated as a method of steatotic liver preservation and has shown promising results. Bessems *et al*[39] compared the traditional method of cold storage *vs* hypothermic MP for rat donor steatotic liver preservation. After 24 h of either hypothermic cold storage or MP, results demonstrated reduced levels of AST and LDH and increased bile production, ammonia clearance, urea production, and ATP levels after MP *vs* cold storage.

Vairetti *et al*[40] examined the effects of rat liver preservation using MP at 20 °C in steatotic livers compared to the SCS method of preservation. Results demonstrated that the adenosine triphosphate/adenosine diphosphate ratio and bile production were higher and oxidative stress and biliary enzymes were lower in the machine preservation-treated steatotic livers compared with the SCS method of preservation[40]. Additionally, there was a 2-fold increase in TNF α levels and caspase-3 activity in the SCS steatotic livers compared with the machine perfused livers[40]. These findings suggest that MP at 20 °C improves rat steatotic liver preservation compared with the SCS method.

Subnormothermic machine preservation has also been investigated in the context of macrosteatotic rat livers and has shown to reduce parenchymal ALT, mitochondrial glutamate dehydrogenase release while protecting against steatotic-induced sinusoidal microvascular alterations and preserving mitochondrial structure[41]. Thus, implementation of defatting protocols seeks to efficiently decrease the proportion of macrosteatotic hepatocytes while ensuring viability and functionality in the remaining hepatocytes[42].

In an effort to mitigate the detrimental effects of hepatic steatosis on transplantation outcomes, several animal studies have investigated the role of defatting protocols to reduce the intrahepatic TAG content prior to transplantation. These protocols may span a period of days to weeks and rely on a change in diet to alter the fat content in livers prior to transplantation.

The concept of defatting steatotic livers holds significant therapeutic and clinical potential as defatting in humans has shown to decrease steatosis. For example, one studied investigated the implementation of a protein-rich (1000 kcal/d) diet, exercise (600 kcal/d), and the lipid-lower drug bezafibrate (400 mg/d) for 2-8 wk in 11 candidates for living-donor liver transplantation[43]. Results demonstrated significantly improved body weight, BMI, and steatosis allowing for the transplantation of 7 of the treated liver grafts to recipients[43]. Post-transplant tests demonstrated liver functioning with no significant differences in measured functional parameters[43]. Additionally, a study of 120 consecutive living donors with non-alcoholic fatty liver disease of ≥ 30% or an estimated donor-recipient weight ratio of < 0.8 demonstrated that following diet and exercise modifications leading to ≥ 10% total cholesterol reduction and ≥ 5% weight reduction, an improvement in steatosis of ≥ 20% was seen in the 120 donors[44].

The aforementioned studies demonstrate the feasibility of reducing fat content using established defatting protocols that are reliant on a diet change prior to liver transplantation, however they do not reflect a viable approach for liver grafts that are procured and intended for transplant, typically requiring a time frame of less than 12 h[45,46]. While the use of defatting protocols to reduce steatosis in donor livers for transplantation has provided initial promising results for expanding the liver donor pool, further experimental animal studies investigating the use of *ex vivo* perfusion of donor livers to reduce steatosis remains limited.

Jamieson *et al*[47] demonstrated in a porcine model that agents involved in peroxisome proliferation for lipid export, visfatin to reduce triglyceride (TG) levels, and forskolin to stimulate oxidation of lipids and ketogenesis decreased hepatocyte TG levels by 31% in 48 h of normothermic MP. Periportal hepatocytes were "defatted" compared to hepatocytes near the perivenous region with an overall increase in bile production[47]. Additionally, Nagrath *et al*[48] utilized a perfusate medium supplemented with defatting agents (forskolin, GW7647, hypericin, scoparone,

visfatin, and GW501516) on steatotic livers from obese Zucker rats. Following *ex vivo* normothermic perfusion for 180 min with the defatting perfusate medium, the TG content decreased by 65% and produced elevated bile levels compared to the control perfusion[48].

Liu *et al*[49] reported the use of subnormothermic (20 °C) MP supplemented with a defatting cocktail for 6 h in obese Zucker rats. Results demonstrated a significant increase in very low density lipoprotein (VLDL) and TG content in the perfusate in groups with and without the defatting cocktail. Additionally, the oxygen uptake rate, VLDL and TG secretion, and venous resistance were also similar in both groups[49]. This study demonstrates the process of lipid export during subnormothermic MP.

In another study, the addition of carvedilol, a beta- and alpha-adrenergic blocking agent, used commonly in the setting of ischemic heart disorders and hypertension, to UW solution prevented rat hepatic injury associated with IRI in both steatotic and non-steatotic livers after 2 h of normothermic perfusion[50-52].

As basic science methods aimed at reducing hepatic steatosis prior to transplantation are constantly being explored and optimized, clinical studies investigating the role of defatting cocktails in MP is relatively limited. Since the field of liver transplantation has only recently established MP as the superior preservation method compared to SCS, we expect more clinical studies focused on the addition of therapeutic agents to the perfusate, such as defatting cocktails, in the near future.

Boteon *et al*[53] has recently utilized pharmacological agents in the setting of *ex situ* normothermic MP to enhance lipid metabolism in steatotic human donor livers discarded for transplantation. Using a previously published cocktail of drugs by Nagrath *et al*[48] and supplemented with L-carnitine, Boteon *et al*[53] added this defatting cocktail to the perfusate at normothermic conditions (37 °C). Results showed that within 6 h of NMP supplemented with the pharmacologic defatting cocktail, the steatotic livers had enhanced lipid metabolism with decreased TG content, decreased vascular resistance of the portal vein with increased flow, decreased lactate levels, and decreased tissue expression of markers implicated in IRI such as CD14 and CD11b and decreased cytokine profiles of TNF-α and IL1β[53]. Most notably, following pharmacologic NMP treatment, all 5 treated livers were considered transplantable based upon viability criteria established by the authors[48,53].

This particular study highlights the potential power of therapeutics administered during MP to salvage previously deemed untransplantable livers while addressing the liver donor pool shortage. To our knowledge, there exists no clinical trial to date using defatting protocols with NMP in the setting of human liver transplantation, as the pharmacological defatting agents used at the bench have not yet been approved for use in humans in a clinical setting[54]. While Boteon *et al*[55] report cytotoxicity results of the defatting cocktail in the setting of primary human hepatocytes on non-parenchymal liver cells, an exciting future for approved pharmacologic defatting therapeutics for human liver perfusion and transplantation surely exists.

Vasodilator administration during liver machine preservation

While IRI induces damage at the cellular level as highlighted, microvasculature is also disrupted. IRI insult affects endothelial cells by disrupting the normal barrier function, vascular tone, and expression of adhesion molecules[56]. Nitric oxide is reduced during reperfusion and can therefore no longer control vasodilation during this period[56]. Additionally, capillaries become occluded due to the activation of inflammatory and coagulation cascades[56]. Therefore, several labs have examined therapeutic agents in MP to preserve the microvascular integrity of the liver.

In a study designed to address the susceptibility of uncontrolled non-heart-beating donors to warm IRI during liver transplantation, the authors devised a new rat model technique for liver grafts using short oxygenated warm *ex vivo* perfusion (SOWP) and prostaglandin E1 (PGE1)[57,58]. PGE1 has been shown to have vasodilative and hepatoprotective effects such as reducing hepatocytic degeneration, central and portal ICAM-1 expression, central and sinusoidal VCAM-1 expression, central and portal P-selectin expression, and portal and sinusoidal E-selectin expression in the context of reperfusion[57]. Results from the SOWP and PGE1 study showed increased total bile production during reperfusion to the same level as the heart-beating donor grafts[58]. Additionally, PGE1 supplementation to the SOWP buffer decreased AST, ALT, and TNFα levels following 1 h of reperfusion[58]. Necrosis and apoptosis were examined *via* histology and TUNEL staining and demonstrated significantly reduced levels following PGE1 treatment with SOWP[58]. This study revealed that SOWP and PGE1 treatment before cold preservation improves the functioning of liver grafts following warm IRI[58].

In a similar SOWP study, Maida *et al*[59] performed rat liver transplantations

following a 6 h cold preservation period in order to determine the *in vivo* effects of SOWP supplemented with PGE1 in DCD rats. Results indicated that in the PGE1-treated SOWP groups, serum liver enzymes, cellular damage, and intercellular adhesion molecule 1 levels were significantly decreased compared to the control group[59].

Nassar *et al*[60] investigated the role of a prostacyclin analog, epoprostenol sodium, in a pig DCD model during NMP for 10 h after 60 min of warm ischemia time and demonstrated lower levels of AST, ALT, LDH, increased bile production, and preserved hepatic architecture compared with the control groups. Echeverri *et al*[61] studied the safety and efficacy of BQ123 (endothelin1 antagonist), epoprostenol (prostacyclin analogue), and verapamil (calcium channel antagonist) in a pig transplantation model using normothermic *ex vivo* liver MP. Livers treated with BQ123 and verapamil demonstrated increased hepatic artery flow and reduced hepatocyte injury compared with controls[61].

Addition of vasodilators to the perfusate solution during MP in animal transplantation models demonstrates significant therapeutic potential. While IRI incites damage to the microvasculature, vasodilators such as PGE1 impart protective effects on the vasculature such as preserving adhesion and selectin expression for maintenance of vascular integrity.

Bae *et al*[62] have also explored the use of α-tocopherol with Vasosol in a DCD rodent model as an additive in the perfusate during HMP to reduce inflammatory and apoptotic markers implicated in reperfusion injury. Addition of α-tocopherol to the HMP solution reduced ALT levels during reperfusion and also reduced levels of inflammatory cytokines IL-6, TNF-α, and MCP-1, and caspase 3/7 as a result of reducing cytochrome C mRNA levels[62].

Clinical trials investigating the role of vasodilator additives to machine perfusate also highlight the importance of therapeutics in the human liver transplantation setting. The first human clinical trial using HMP for liver transplantation was completed by Guarrera *et al*[63] and compared HMP-preserved livers in 20 adults with a SCS matched group[45,63,64]. A Vasosol solution was used as the HMP perfusate which included added antioxidants, metabolic substrates, and vasodilators including nitroglycerin and prostaglandin E1[45,63]. Results indicated significantly reduced peak levels of AST, ALT, total bilirubin, and serum creatinine in the HMP preserved group. Molecular analysis from this clinical study revealed that HMP attenuated expression of inflammatory cytokines, oxidation markers, adhesion molecules and chemokines, and apoptosis and CD68 positive macrophages compared with the SCS group[64]. The overall early graft dysfunction rates were 5% in the HMP groups compared with 25% in the control group, and the mean hospital stay was also shorter in the HMP group compared with the SCS group suggesting that HMP with vasodilator additives in liver transplantation is a safe and future method of liver perfusion and preservation[45].

OTHER THERAPEUTICS IMPLEMENTED IN LIVER MP

Additional classes of therapeutics used in the setting of liver MP include anti-inflammatory agents, human liver stem cell-derived extracellular vesicles (HLSC-EV), and more recently, the δ-opioid agonist, enkephalin. While these therapeutics will not be discussed extensively in this review, they are summarized in Table 2 and are important to mention given their potential therapeutic role in the liver transplantation field.

Goldaracena *et al*[9] reported improved liver function and lower inflammation in a pig transplantation model using anti-inflammatory agents (alprostadil, n-acetylcysteine, carbon monoxide, sevoflurane) added to perfusate in NMP. Rigo *et al*[65] demonstrated, for the first time, reuptake of HLSC-EV in *ex vivo* rat liver perfusion. Treated livers demonstrated reduced necrosis and apoptosis, lower hypoxia-induced markers, and superior liver function (lower AST and LDH).

Recently, Beal *et al*[66] demonstrated the efficacy of enkephalin, a δ-opioid agonist, to reduce oxidative stress in a rat *ex vivo* perfusion model. Treated livers had lower AST and malondialdehyde levels, in addition to higher ATP and glutathione levels in the perfusate. Yu *et al*[67] also most recently reported that the selective NLRP3 inflammasome inhibitor mcc950 added to the perfusate of an HMP system and intravenously injected in a pig liver transplantation model demonstrated improved outcomes in DCD organs compared with controls.

Table 2 Other major therapeutic additives in models of *ex vivo* liver machine perfusion

Ref.	*Ex vivo* perfusion type	Therapeutic	Problem	Animal	Model	Sample size	*Ex vivo* perfusion time (h)	Outcomes
Goldaracena *et al*[9], 2016	SNMP	Anti-inflammatory agents (Alprostadil, n-acetylcysteine, carbon monoxide, sevoflurane)	IRI	Pig	Transplantation	5	4	During EVLP: lower AST, TNF-α, IL-6 Lower HA levels, β-galactosidase and higher IL-10 (nonsignificant) After transplantation: Lower bilirubin, lower IL-6, lower cleaved caspase 3 staining, intact sinusoidal endothelial cell lining Lower AST, TNF-α, HA, ALP and higher IL-10 (nonsignificant)
Rigo *et al*[65], 2018	NMP	HLSC-EV	IRI	Rat	EVLP	9	4	HLSC-EV uptake in treated livers Reduced necrosis and apoptosis on histology, lower Suzuki tissue injury score, lower apoptosis, lower AST and LDH, lower HIF-1α & TGF-β1 (hypoxia induced markers) NMP had low hematocrit to induce hypoxia
Beal *et al*[66], 2019	NMP	Enkephalin (δ-opioid agonist)	IRI	Rat	EVLP	6	4	10 μmol/L determined to be optimal concentration in an *in vitro* model: Lower ALT and MDA; better preservation of structural architecture; decreased caspase-3 expression; decreased TUNEL staining; decreased phosphorylation of p38 and JNK; increased expression of p-Akt, PI3K, p-Bad and Bcl-2
Yu *et al*[67], 2019	HMP	NLRP3 Inflammasome Inhibitor mcc950	IRI	Pig	Transplantation	6	2	All reduced in HMP-postop group with added mcc950 in perfusate and IV administration of mcc950 After transplantation: TNF-α, IL-1β, β-galactosidase, post-reperfusion serum ALT and AST, MDA, apoptosis staining, caspase-1 levels

SNMP: Subnormothermic machine perfusion; NMP: Normothermic machine perfusion; HMP: Hypothermic machine perfusion; IRI: Ischemia-reperfusion injury; EVLP: *Ex vivo* liver perfusion; AST: Aspartate aminotransferase; HA: Hyaluronic acid; ALP: Alkaline phosphatase; HLSC-EV: Human liver stem cells-derived extracellular vesicles; LDH: Lactate dehydrogenase; ALT: Alanine aminotransferase; MDA: Malondialdehyde; IV: Intravenous.

CONCLUSION

The use of administered therapeutics during machine liver perfusion has demonstrated promising results in basic science studies and initial clinical reports indicate their safety and efficacy in human liver transplantation. While novel therapeutic approaches to combat IRI are being developed through basic science research, their use in clinical medicine and treatment in patients for liver transplantation has yet to be fully explored. The number of human clinical trials investigating the use of liver MP is increasing, however the information obtained from these trials remains limited until additional robust studies are performed. A proposed summary of the advantages and limitations of the aforementioned MP therapeutics is offered in Table 3. While the therapies mentioned are relatively recent advances in the

Table 3 Summary of proposed advantages and limitations of liver machine perfusion therapeutics

Therapeutic	Advantages	Limitations/future considerations
RNAi pathway	Selectively targets and silences/degrades specific genes	Most beneficial/effective siRNA target against liver IRI in transplantation remains to be identified
	Mechanism of siRNA silencing pathway is generally understood	Potential for administration of multiple siRNA constructs each with a different target
	Organ-specific uptake	Requires design of siRNA against target mRNA and validation of target silencing
	Permits imaging studies visualizing tissue uptake and distribution	
Defatting cocktails	Ability to restore liver function by defatting	Steatotic livers are already predisposed to IRI[32]
	Aimed at enhancing natural lipid metabolism *via* lipid export, reduction of triglyceride levels, and stimulation of lipid oxidation and ketogenesis	Mechanisms of glucose and lipid control in liver remain poorly defined[69]
		Undefined consensus for quantifying degree of steatosis[70]
		Need for perfusate exchange protocol as secreted triglycerides recirculate causing further increase in lipid deposition[47]
		Kinetics of defatting may surpass average timeframe of liver transplantation[48]
Vasodilators	Focused on improving intrinsic function of liver to improve blood flow *via* smooth muscle relaxation and vasodilation	Effects of vasodilators in marginal grafts remains unclear[61]
	Act to increase arterial flow and decrease post-sinusoidal resistance[71]	Combination of agents does not allow for specific identification of most beneficial agent(s)
Anti-inflammatory agents	Some agents also act as vasodilators[68,72,73]	Mechanisms of anti-inflammatory agents remains unexplored in context of *ex vivo* liver perfusion[9]
	Act to scavenge free radicals to prevent IRI[74]	Combination of agents does not allow for determination of which specific agents were beneficial[9]
	Ability to protect other cell types such as endothelial cells[75,76]	
HLSC-EV	Regenerative and hepatoprotective properties[77,78]	Unknown mechanism of hypoxic protection[65]
	Diverse differentiating capabilities[77]	Timing of EV uptake during NMP currently unknown[65]
	Contain mRNA and miRNA subsets that modulate activity of target cells[79]	
	May serve as option for liver diseases without need for stem cells transplantation[65]	
δ-opioid agonist (Enkephalin)	Protects against oxidative stress[66]	Unknown therapeutic role in setting of post-perfusion liver transplant model with measured outcomes of graft function[66]
	Prevention of mitochondrial dysfunction *via* opioid receptor signaling[66]	
	Protection against IRI by slowing cellular metabolism[80,81]	Unknown role in cold ischemia conditions for liver transplant models[66]
NLRP3 inflammasome inhibitor (mcc950)	Blocks NLRP3-inflammsome activation preventing inflammatory liver damage[82,83]	mcc950 half-life is 3.27 h, while NLRP3 inflammasome activation lasts for several days after reperfusion[84,85]
	Reduces apoptosis post liver transplantation[67]	Additional mcc950 inhibition studies involving *in vitro* and *in vivo* models needed[67]

HLSC-EV: Human liver stem cells extracellular vesicles; NLRP3: Nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3.

field of *ex vivo* liver MP, potential future directions are also included in Table 3 to indicate areas of further exploration. It is also worth noting that in terms of future considerations, the aforementioned studies in this review commonly employed multiple therapeutic agents in the perfusion solution, and several studies highlighted the need to delineate which agents out of the administered cocktail indeed conferred the most protective effect on the liver during *ex vivo* perfusion. MP therapeutics in liver transplantation therefore has tremendous potential to increase the liver donor pool and decrease the waiting time for lifesaving organs.

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REVIEW

Machine perfusion in abdominal organ transplantation: Current use in the Netherlands

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Abstract

Scarcity of donor organs and the increment in patients awaiting a transplant increased the use of organs from expanded criteria donors or donation after circulatory death. Due to the suboptimal outcomes of these donor organs, there is an increased interest in better preservation methods, such as *ex vivo* machine perfusion or abdominal regional perfusion to improve outcomes. This state-of-the-art review aims to discuss the available types of perfusion techniques, its potential benefits and the available evidence in kidney, liver and pancreas transplantation. Additionally, translational steps from animal models towards clinical studies will be described, as well as its application to clinical practice, with the focus on the Netherlands. Despite the lack of evidence from randomized controlled trials, currently available data suggest especially beneficial effects of normothermic regional perfusion on biliary complications and ischemic cholangiopathy after liver transplantation. For *ex vivo* machine perfusion in kidney transplantation, hypothermic machine perfusion has proven to be beneficial over static cold storage in a randomized controlled trial, while normothermic machine perfusion is currently under investigation. For *ex vivo* machine perfusion in liver transplantation, normothermic machine perfusion has proven to reduce discard rates and early allograft dysfunction. In response to clinical studies, hypothermic machine perfusion for deceased donor kidneys has already been implemented as standard of care in the Netherlands.

Key words: Machine perfusion; Review; Kidney transplantation; Liver transplantation; Pancreas transplantation

Core tip: Scarcity of donor organs and the increment in waitlisted patients increased the use of organs from expanded criteria donors or donation after circulatory death donors. Due to suboptimal outcomes of these organs, there is an increased interest in dynamic preservation, such as *ex vivo* machine perfusion or abdominal regional perfusion to

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improve outcomes. This review discusses perfusion types, its potential benefits and the available evidence in kidney, liver and pancreas transplantation. Additionally, translational steps from animal models towards clinical studies will be described as well as its application to clinical practice, with as focus the Netherlands.

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INTRODUCTION

The major obstacle in organ transplantation is the imbalance between demand and supply of suitable donor organs. In the Eurotransplant region, a total number of 14773 patients were on the active organ waiting list on 1 January 2018, while only a number of 7918 patients received a transplant from either a living or deceased donor[1,2]. Consequently, approximately 50% of the waitlisted patients did not receive an organ transplant and either remained waitlisted, became unfit for transplant or died while being waitlisted. This accentuates the urgent need to tackle organ shortage. One solution to address this problem is the increased use of suboptimal organs, such as organs from expanded criteria donors (ECD) or donation after circulatory death (DCD). However, DCD donation is not performed in several countries, mainly because of legal restrictions. Besides, ECD and DCD organs have impaired clinical outcomes based on their poor tolerance to ischemia-reperfusion injury[3]. The outcomes of DCD kidney, liver and pancreas transplantation in comparison to donation after brain death (DBD) have been previously described and are summarized in Table 1. DCD kidneys are more prone to delayed graft function (DGF) and primary non function (PNF), while graft survival is similar[4,5]. DCD livers have more frequent biliary complications, such as ischemic cholangiopathy, with corresponding inferior graft and patient survival rates[6]. For pancreas transplants from a DCD donor, the odds of graft thrombosis are 1.67 times higher compared to DBD pancreas transplants[7]. Therefore, increasing the quality of those suboptimal organs is of paramount importance.

The best strategy for organ preservation in an era where the use of ECD and DCD organs continues to increase is still a major topic of discussion. During the past decade, there has been renewed interest in the use of machine perfusion instead of static cold storage (SCS) as a preservation technique. The concept behind machine perfusion is dynamic reconditioning and repair through restoring blood flow of the donor organ by connecting it to a pump with the possibility to add oxygen and therapeutic agents. Besides this benefit of organ repair that may lead to improved organ quality, machine perfusion has the promising possibility to make initially discarded organs transplantable[8-10]. The second benefit is the possibility of pre-transplantation viability assessment of the donor organ "while on the pump" to prevent unnecessary transplantations with an organ that will never function in the recipient[11,12]. The third benefit is the possibility to extend the time until transplantation, for example in order to provide daytime surgery and to allow time for transfer of the donor organ to the recipient hospital.

The Netherlands has a continuously growing abdominal transplantation program, as shown in Figures 1-3. In the past years, there has been an extensive increase in the DCD program. For kidney transplantation, the amount of DCD organs transplanted within the deceased donor organ transplant program was 39% in 2009, and this increased up to 55% in 2018[13,14]. For the DCD liver transplant program, this was 23% in 2009, which increased up to 39% in 2018[13,14]. For pancreas transplantation, the amount of DCD grafts used increased from 0% in 2009 to 42% in 2018[13,14]. So far, many experimental studies show the potential beneficial effects of machine perfusion in various types of organ transplantation. Many clinical studies have been recently published, translating the earlier experimental work into the clinic. Standard of care concerning organ preservation in the Netherlands already changed in response to earlier published clinical studies. With this state-of-the-art review, we aim to describe the history of machine perfusion in abdominal organ transplantation, as well as the rationale behind different types of perfusion, its potential benefits and its current use in the Netherlands as one of the pioneering countries with regard to translating

Table 1 Outcomes from meta-analyses or large studies comparing donation after circulatory death to donation after brain death outcomes in abdominal organ transplantation

	DCD	DBD	*P* value
Kidney[56]			
PNF (%)	3.2	2.6	0.06
DGF (%)	48.5	24.9	< 0.001[a]
1-yr eGFR[1]	47.4 (35.6-61.2)	48.7 (37.3-61.1)	0.69
5-yr graft survival (%)	76.8	78.1	0.60
5-yr patient survival (%)	86.5	89.4	< 0.001[a]
Liver[57]			
Biliary complications (%)	26	16	< 0.001[a]
Ischemic cholangiopathy (%)	16	3	< 0.001[a]
3-yr graft survival (%)	73	74	0.01[a]
3-yr patient survival (%)	82	88	0.04[a]
Pancreas[7]			
Graft survival	HR 0.98 (0.74-1.31)	Reference value	0.92
Patient survival	HR 1.31 (0.62-2.78)	Reference value	0.47
Graft thrombosis	OR 1.67 (1.04-2.67)	Reference value	0.006[a]

[1]Data is presented as median and interquartile range.
[a]Statistically significant. DBD: Donation after brain death; DCD: Donation after circulatory death; DGF: Delayed graft function; eGFR: Estimated glomerular filtration rate; HR: Hazard ratio; OR: Odds ratio; PNF: Primary non function.

machine perfusion in standard of care.

HISTORY

In the 1960s, machine perfusion became part of clinical practice with its main goal to extend preservation time for cross-matching and transportation of the organ[15]. However, in the late 1980s, Folkert Belzer and James Southard[16-18] developed the University of Wisconsin solution, which improved preservation time significantly when compared to the commonly used EuroCollins solution. Because SCS was a much cheaper and simpler manner of organ preservation without compromising donor organ quality, the interest in machine perfusion decreased[19,20].

In the Netherlands, important research steps concerning preservation techniques started with the usage of hypothermic machine perfusion (HMP) on donor kidneys. In 1978, a study was published showing that kidneys severely damaged by ischemia were found to have a higher percentage of immediate function when preserved with HMP compared to SCS[21]. Five years later, an article was published wherein the clinical outcomes of 75 kidneys transplanted after HMP were compared to 2686 kidneys transplanted after SCS in the Eurotransplant region. Creatinine clearance, PNF and DGF did not differ significantly[22]. These studies raised the hypothesis that only kidneys that have been subjected to prolonged periods of warm ischemia might benefit from HMP in an era of mainly standard criteria donors[22,23]. Later on, when organ shortage forced the more frequent use of ECD donors, various clinical studies were published suggesting that HMP could result in better short-term outcomes, especially in those ECD donors[24-26]. This led to the Machine Perfusion Trial, a randomized controlled trial (RCT) executed in the Eurotransplant region with the University Medical Center Groningen as principal investigator. The results, published in 2009, showed that HMP was associated with a reduced risk of DGF and improved graft survival in the 1st year after transplantation[27].

Research concerning normothermic machine perfusion (NMP) started in the early eighties. Two important animal studies concerning normothermic *ex vivo* perfusion were carried out, also with the goal to allow longer preservation times[28,29]. The first study was carried out in a dog auto transplant model. Twenty-four dogs were assigned to one of four intervention groups, differing in total preservation time (96 h or 144 h) and HMP alone or interrupted with 4 h of normothermic perfusion on the animal. For both preservation times, the two groups (2 and 4) who also underwent normothermic perfusion had significantly higher creatinine clearance than the HMP only group. This suggested that interruption of HMP by normothermic perfusion

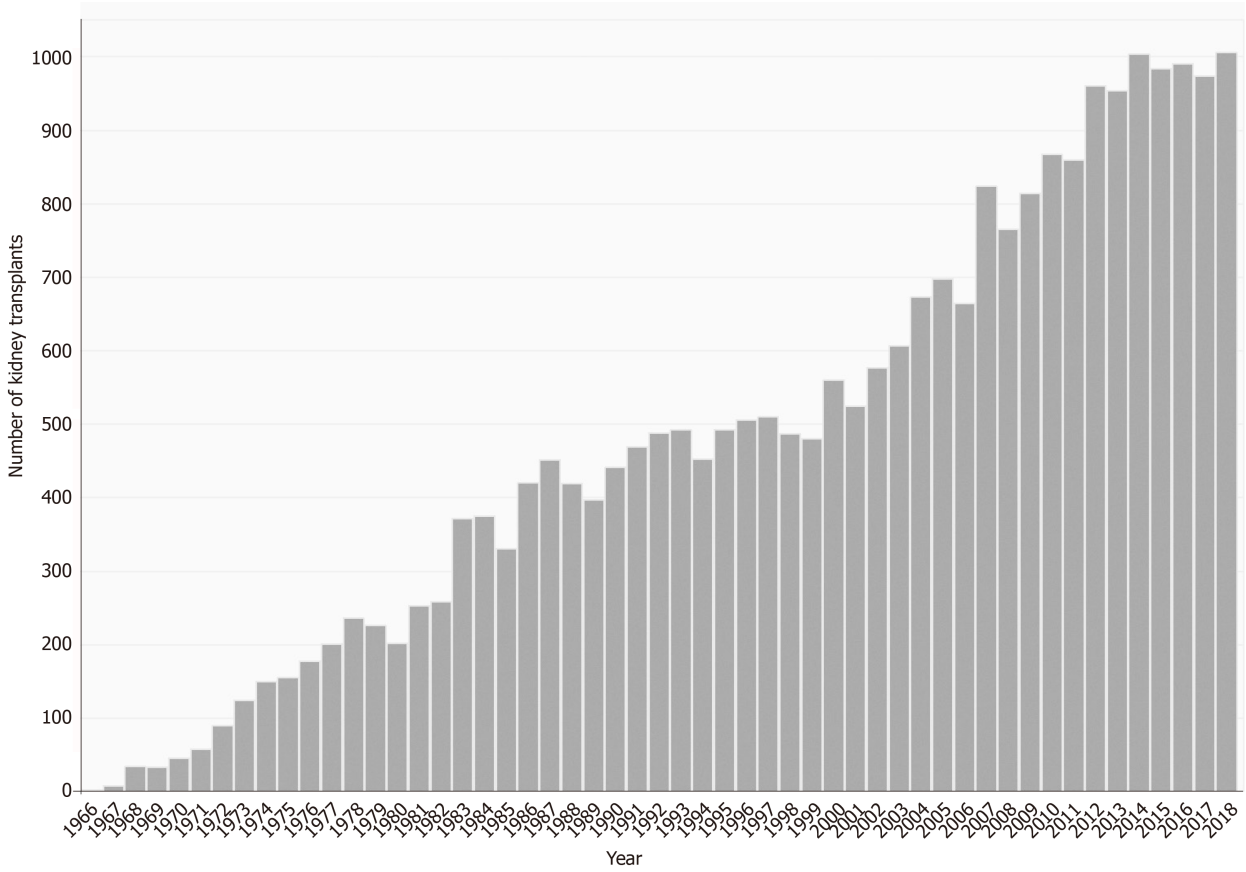

Figure 1 Number of kidney transplantations in the Netherlands per year

improves results, which was later also confirmed in a rabbit study[28,29]. In 2002, Brasile *et al*[30] investigated graft function in a canine auto-transplant model using kidneys with a prolonged warm ischemia time. He found that all kidneys after NMP had direct function, in contrast to kidneys transplanted after HMP or SCS. NMP became of larger interest due the increased use of ECD organs. The hypothesis was that those organs require careful reconditioning and repair, which may not be optimal in a hypothermic environment where metabolism is suppressed. Together with the growth in the amount of ECD donors, NMP gained interest, with the first in human kidney transplantation after NMP in 2011[31]. Currently, only small clinical studies have been performed concerning NMP. An RCT comparing NMP to SCS in DCD kidney transplantation is currently ongoing in the United Kingdom, with the expected results to be published in 2020/2021 (ISRCTN15821205).

ABDOMINAL REGIONAL PERFUSION

Rationale of ARP

The development of ARP took place in Spain among uncontrolled DCD donors in a successful attempt to increase the donor pool. Abdominal regional perfusion (ARP), depending on the temperature also called normothermic regional perfusion (NRP) or hypothermic regional perfusion (HRP), is an *in-vivo* dynamic preservation technique that is performed while the organs are still in the donor. After withdrawal of life-sustaining support and circulatory arrest with a following period of no-touch, the donor is transferred to the operating room. In the non-ARP situation, the super-rapid recovery (SRR) technique is used to access all potential donor organs and cannulate the aorta to start cold flushing as quickly as possible, which ends the first warm ischemia time. Then, the donor organs will be inspected on eligibility for transplantation and the organs will be retrieved. In the ARP situation, cannulas will be placed in either the abdominal aorta and caval vein or in the femoral artery and femoral vein. The cannulas are connected to an extracorporeal membrane oxygenation-like device, which uses a pump to recover donor venous blood, oxygenate it and add substrates. Subsequently, the oxygenated blood will be returned

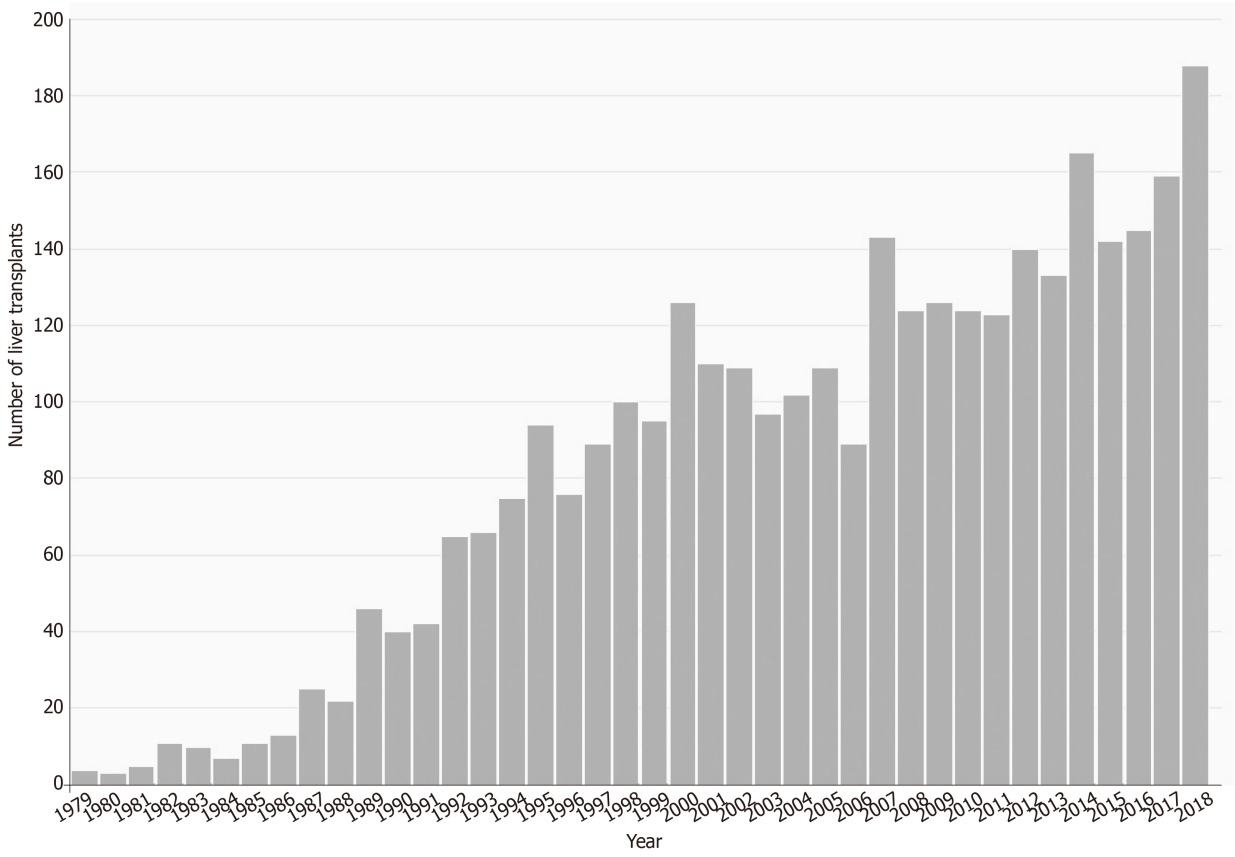

Figure 2 Number of liver transplantations in the Netherlands per year.

to the subdiaphragmatic aorta. The thoracic aorta is clamped to prevent auto-resuscitation, which has been shown to be an effective method[32]. Depending on the law regulations per country, cannulation into the femoral vein and artery is to be performed before cardiac arrest to reduce further warm ischemia time. However, in most countries in Europe, no pre-mortem interventions are allowed by law. Besides the hypothesis that ARP improves organ quality by minimizing warm ischemia time, there are more benefits of this technique. Because the organs are still in the donor body, this creates a more physiological environment for the organs than connected to a pump outside of the body. Also, it is possible to perform viability assessment in the donor. As a third reason, ARP modifies an urgent procedure into an elective organ recovery procedure, which could reduce organ damage and organ losses due to surgical events. Also, NRP is supposed to be more cost-effective than NMP because multiple organs are resuscitated through one procedure.

Clinical outcomes after ARP

Kidney: Literature concerning kidney transplantation after ARP is scarce, and most of the literature focuses on NRP instead of HRP. Table 2 contains the core clinical studies describing clinical outcomes of kidney transplantation after ARP. A few studies also compared ARP outcomes to either DBD outcomes or outcomes after retrieval with the SRR technique. Farney *et al*[33] compared 25 kidney transplants after HRP to kidney transplants retrieved with the SRR technique. They concluded that kidney transplants after HRP had lower rates of DGF and shorter hospitalization. Lee *et al*[34] investigated 31 kidney transplant outcomes after HRP and compared those to outcomes after DBD or living donor kidney transplant. He showed a higher rate of DGF in comparison to DBD but similar incidence of acute rejection and 5-year graft and patient survival rates. Valero *et al*[35] described *in situ* perfusion with HRP and NRP. They concluded a lower incidence of DGF and PNF after NRP when compared to *in situ* perfusion or total body cooling[35]. Miñambres *et al*[36] investigated 37 kidney transplantations after NRP and compared their clinical outcomes to DBD kidney transplant outcomes. They showed that graft survival was similar to graft survival of a DBD kidney with 5% PNF and 27% DGF[36]. Also, Magliocca *et al*[37] compared the outcomes after NRP with DBD outcomes. They concluded no statistically significant differences in DGF, PNF and rejection. In conclusion, HRP could possibly reduce the incidence of DGF and hospitalization duration after kidney transplantation when compared with the SRR

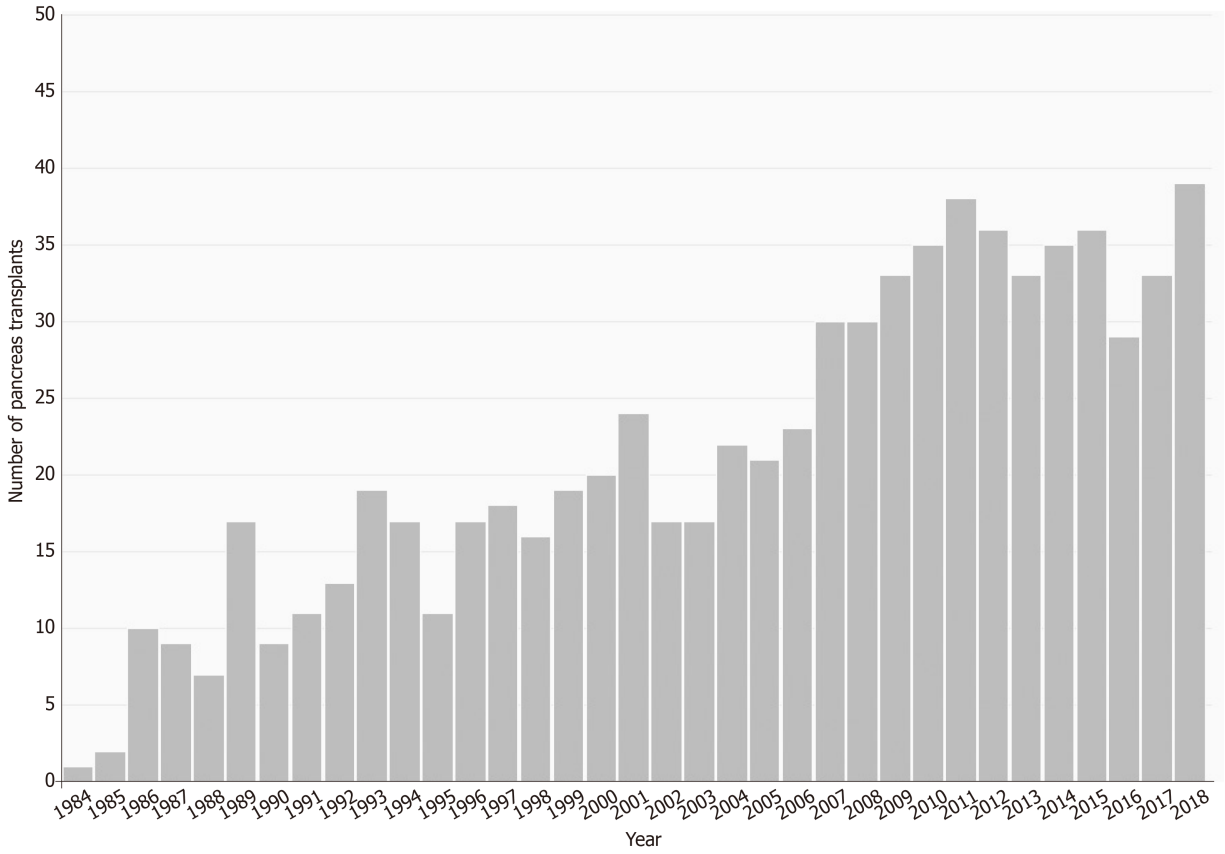

Figure 3 Number of pancreas transplantations in the Netherlands per year.

technique. Graft survival after NRP resembles DBD graft survival, which has been shown to be similar to DCD kidney graft survival using the SRR technique[5]. However, NRP may reduce the incidence of DGF and PNF.

Liver: Only incidental cases have been described concerning liver transplantation after HRP[33,38,39]. The outcomes of those transplants are not discussed separately. The results from liver transplantation after NRP are summarized in Table 3. One study by Hessheimer *et al*[40] compared 95 controlled DCD liver transplant outcomes after NRP to the outcomes of liver transplantation after retrieval with the SRR technique. They showed a significant decrease in favor of the NRP group for graft loss (NRP: 12%, SRR: 24%), biliary complications (NRP: 8%, SRR: 31%), ischemic cholangiopathy (NRP: 2%, SRR: 13%) and retransplantation rates (NRP: 5%, SRR: 9%). Miñambres *et al*[36] and Fondevila *et al*[41] described NRP-DCD liver transplant outcomes in comparison to DBD outcomes. They concluded that graft and patient survival after NRP-DCD liver transplantation is comparable to DBD liver transplantation. In conclusion, NRP decreases the incidence of biliary complications, ischemic cholangiopathy, graft loss and retransplantation rates when compared to the SRR technique. Graft and patient survival rates are comparable to those after DBD liver transplantation.

Pancreas: There is scarcity of studies about pancreas transplant outcomes after the use of ARP. Two aforementioned studies for kidney and liver transplant outcomes after NRP also described some anecdotal cases of pancreas transplant outcomes after NRP. Oniscu *et al*[42] described two combined kidney-pancreas transplants and one islet transplant after NRP, all with primary function. Miñambres *et al*[36] described one combined kidney-pancreas transplant after NRP, also with primary function. Butler *et al*[43] described two pancreas transplants after 120 minutes of NRP, both with primary function.

Current practice in the Netherlands

In October 2018, the first liver transplantation after NRP was successfully performed in Erasmus Medical Center, Rotterdam, The Netherlands. This transplantation was part of the NRP project, a collaboration between organ retrieval team West (consisting of Leiden University Medical Center and Erasmus Medical Center) and subsidized by

Table 2 Clinical studies published about kidney transplant outcomes after abdominal regional perfusion

Study	*n*	DCD type	Rejection, %	DGF, %	PNF, %	Graft survival, %			Patient survival, %		
						1	3	5	1	3	5
HRP											
Valero *et al*[35], 2000	8	II	-	75	0	-	-	-	-	-	-
Koyama *et al*[58], 2002	46	III/IV	-	87	6.5	88.3	-	-	-	-	-
Lee *et al*[34], 2005	31	II/III/IV	35.5	41.9	0	100	-	88.4	100	-	100
Sánchez-Fructuoso *et al*[59], 2006	320	I/II	4.4	60.9	4.4	87.4	-	82.1	95	-	90
Farney *et al*[33], 2011	25	III	16	21	0	88	88	-	-	-	-
NRP											
Valero *et al*[35], 2000	8	II	-	12.5	0	-	-	-	-	-	-
Maglioccaet *al*[37], 2005	24	III	0	8.3	0	-	-	-	-	-	-
Reznik *et al*[60], 2011	20	II	10	70	10	-	-	-	-	-	-
Hessheimer *et al*[61], 2015	158	II	-	65	9	88	-	-	-	-	-
Oniscu *et al*[42], 2014	32	III	-	40	6	87.5	-	-	96.8	-	-
Butler *et al*[43], 2014	14[1]	III	-	18.2	9.1	-	-	-	-	-	-
Rojas-Peña *et al*[62], 2014	29[2]	III	-	31	3.5	-	-	-	-	-	-
Demiselle *et al*[63], 2016	19	II	-	53	5.3	94	-	-	100	-	-
Miñambres *et al*[36], 2017	37	III	-	27	5	91.8	-	-	-	-	-

[1]Fourteen kidneys were transplanted in 11 recipients. Therefore, clinical outcomes are calculated in the 11 recipients;
[2]Outcomes were only mentioned from their own center. DCD: Donation after circulatory death; DGF: Delayed graft function; HRP: Hypothermic regional perfusion; NRP: Normothermic regional perfusion; PNF: Primary non function.

the Ministry of Health, Welfare and Sport. The goal of this project is to increase the number of transplantable organs and to improve organ quality. NRP can be carried out in every potential DCD donor, but within the Dutch project it is currently only carried out within DCD type III donors. Different protocols exist in the literature for pump parameters during NRP. In the Dutch NRP project, a pump flow of 2-3 L per minute is pursued with a temperature starting at 33 °C that is slowly increased to 37 °C. For oxygen, a mix between air and oxygen is used with the aim to reach a PaO_2 of 110-150 mmHg. Loss of volume is supplemented by adding red blood cells concentrate, albumin and Ringer's lactate. The circuit is primed with heparin to prevent the blood from clotting. Bicarbonate is added in case of acidosis to keep the pH within a physiologic range. For the liver, the following issues are considered to determine suitability for transplantation: (1) Aspartate aminotransferase (ALAT) less than 4 times the upper limit at the end of NRP; (2) ALAT reaches its plateau phase between first and second hour; (3) Lactate below 5 mmol/L at the end of NRP; (4) Glucose doubles at the end of NRP in comparison to the start of NRP and (5) Glucose is above 10 mmol/L at the end of NRP. After 2 years, results of this project will be analyzed to see whether this technique should be implemented nationwide in the Netherlands.

EX VIVO MACHINE PERFUSION

Rationale of ex vivo machine perfusion

Whereas the goal of preserving an organ on SCS is slowing down deterioration of the donor organ, the goal of *ex vivo* machine perfusion is sustaining organ viability, organ repair and organ preconditioning. This all takes place in the period between procurement and transplantation of the donor organ with the main goal to optimize outcomes of the graft when transplanted in the recipient. In comparison to ARP, *ex vivo* machine perfusion takes place after organ retrieval, and it may also be used in case of a DBD donor organ. During *ex vivo* machine perfusion, the donor organ is connected to an often pressure-controlled perfusion device that pumps perfusate solution continuously through the organ vasculature. *Ex vivo* machine perfusion can be performed at different temperatures: Hypothermia, normothermia and subnormothermia. In comparison to SCS, HMP may be a more efficient way of cooling of the donor organ while metabolic and toxic waste products are washed out.

Table 3 Clinical studies published about liver transplant outcomes after normothermic regional perfusion

Study	*n*	DCD type	Rejection, %	BC, %	IC, %	PNF, %	Graft survival, %			Patient survival, %		
							1	3	5	1	3	5
Otero *et al*[64], 2004	14	II	22	-	28	28	43	-	-	71	-	-
Fondevila *et al*[41], 2007	10	II	-	10	-	10	50	-	-	70	-	-
Jiménez-Galanes *et al*[65], 2009	20	II	-	-	5	10	80	-	-	85	-	-
Fondevila *et al*[66], 2012	34	II	-	12	8	4.3	-	-	-	-	-	-
Oniscu *et al*[42], 2014	11	III	-	18.2	0	9.1	87.5	-	-	96.8	-	-
Butler *et al*[43], 2014	3	III	-	-	0	-	-	-	-	-	-	-
Rojas-Peña *et al*[62], 2014	13	III	-	-	14.3	14.3	85.7	-	-	-	-	-
Hessheimer *et al*[61], 2015	42	II	-	-		10	73	-	-	-	-	-
De Carlis *et al*[67], 2016	7	II/III	14.3	14.3	0	0	-	-	-	-	-	-
Miñambres *et al*[36], 2017	11	III	-	0	0	9.1	90.9	-	-	-	-	-
Hessheimer *et al*[40], 2019	95	III	-	8	2	2	88	88	-	93	93	-

BC: Biliary complications; DCD: Donation after circulatory death; IC: Ischemic cholangiopathy; NRP: Normothermic regional perfusion; PNF: Primary non function.

During NMP, the temperature is within physiologic range, which increases metabolic activity and allows for active repair and reconditioning. Therefore, NMP may be more beneficial in donor organs that require reconditioning, such as ECD organs.

As normothermia leads to metabolic activity, an oxygenated perfusate is essential. Therefore, a blood-based perfusate is often used, containing washed and leukocyte-depleted red blood cells. Another option is to use an acellular perfusion solution containing a hemoglobin-based oxygen carrier. No studies have investigated which of the two is preferred. In practice, the blood-based perfusate is more popular, probably because this option is less expensive. For NMP, additional substances are added to provide the best circumstances for active repair. The composition and number of additives in the perfusate differs. In general, antibiotics, vitamins, prostaglandins, bicarbonate and heparin to prevent thrombosis are added. Currently, there is no evidence favoring one perfusate solution over another. For HMP, kidney perfusion solution-1 is used as the standard solution for clinical machine perfusion, without additional substances.

Clinical outcomes of ex vivo machine perfusion

Kidney: In abdominal organ transplantation, most clinical research concerning *ex vivo* machine perfusion is carried out in kidney transplants. Currently ongoing RCTs or clinical trials involving discarded kidneys are mentioned in Table 4. There is conclusive evidence for the benefits of HMP over SCS. In 2009, the aforementioned Machine Perfusion Trial of the Consortium for Organ Preservation in Europe (COPE) was published, showing that non oxygenated HMP offers a graft survival benefit in comparison to SCS and a decrease in DGF in all deceased donor kidneys[27]. Subsequently, the COPE-COMPARE trial was initiated, investigating the possible beneficial effects of adding oxygen to HMP. The preliminary results as presented on the American Transplant Conference 2019 showed that oxygenated HMP shows a significant benefit for graft survival and 1-year graft function, which is possibly mediated through a lower risk of BPAR[44]. The results from other studies, as mentioned in the Table 4, have not been published yet.

In contrast to HMP, clinical studies concerning the use of NMP in kidney transplantation are still in its infancy. This is possibly because NMP may be more hazardous because potential failure of NMP leads to harmful additional warm ischemia time. In 2011, the first human kidney transplant after *ex vivo* NMP was performed in the United Kingdom with good post-transplant outcomes[31]. Currently, there is no evidence from RCTs yet that NMP may be beneficial. However, experimental studies have already shown the benefits of NMP over HMP[45]. A phase II, multi-center RCT is currently recruiting to assess the efficacy of 1 h *ex vivo* NMP compared to SCS only in DCD III and IV kidney transplantation (ISRCTN15821205). However, this RCT does not answer the question whether the addition of NMP has beneficial effects in comparison to HMP only. Another study from the Cambridge group is assessing the use of NMP in discarded kidneys, with the primary aim to make them transplantable.

Table 4 Currently ongoing clinical trials concerning *ex vivo* machine perfusion in kidney transplantation

Name of study	Registration number	Design	PI	*n*	Primary outcome	Intervention	Included donors	Results
Unknown	ISRCTN91315246	Non-randomized	Cambridge	90	Graft function	1 h NMP	Discarded kidneys	November 2019
COPE-POMP	ISRCTN63852508	RCT	COPE Essen	262	Graft survival 1y	Short period HMP *vs* SCS only	ECD-DBD	July 2019
COPE-COMPARE	ISRCTN32967929	RCT	COPE Leuven	162	Kidney graft function 1 y	HMP with oxygen *vs* HMP without oxygen	DCD III	↓ risk BPAR ↑ 1-y eGFR[44]
PIO	NCT03031067	Case control	Bologna	20	Graft function	2 h HMP *vs* SCS	ECD -DBD	February 2018
PREDICTION	NCT02055950	Case control	Bergamo	60	Kidney function	HMP *vs* SCS	ECD-DBD	Augustus 2018
Unknown	NCT03837197	RCT	Bologna	260	DGF	2 h oxygenated HMP *vs* SCS	ECD-DBD	December 2021
IMPULSION	NCT01170910	RCT	Lyon	162	DGF	6-8 h HMP *vs* SCS	ECD	August 2016
Machine perfusion trial	ISRCTN83876362	RCT	COPE Groningen	654	DGF	Non-oxygenated HMP *vs* SCS	DCD III and DBD	↓ risk of DGF (OR 0.57) ↓ risk of graft failure (HR 0.52) ↑ allograft survival (94 *vs* 90%, P = 0.04)[27]
Unknown	ISRCTN15821205	RCT	Cambridge	400	DGF	1 h pre-transplant NMP *vs* SCS	DCD III and IV	January 2021

BPAR: Biopsy proven acute rejection; COPE: Consortium for Organ Preservation in Europe; DBD: Donation after brain death; DCD: Donation after circulatory death; DGF: Delayed graft function; ECD: Expanded criteria donor; eGFR: Estimated glomerular filtration rate; HMP: Hypothermic machine perfusion; HR: Hazard ratio; NMP: Normothermic machine perfusion; OR: Odds ratio; PI: Principal investigator; RCT: Randomized controlled trial; SCS: Static cold storage.

Liver: Currently, there are many ongoing clinical trials for liver *ex vivo* machine perfusion. Table 5 summarizes all ongoing RCTs and non-randomized trials in discarded livers. Oxygenated HMP, better known as HOPE in the liver transplantation field, is currently under investigation together with RCTs investigating dual hypothermic oxygenated machine perfusion (DHOPE). The results from these RCTs are expected to follow in 2020. A case control study from the Groningen group in 10 patients found a higher graft survival, a two-fold lower peak ALAT and bilirubin in livers treated with DHOPE[46].

There is conclusive evidence for NMP over SCS in donor livers. In 2018, a RCT among all deceased liver donors was published comparing SCS to NMP with a minimal duration of 4 h[47]. This study showed a 49.4% reduced peak ASAT during the first 7 d post-transplant in both DCD and DBD livers[47]. Early allograft dysfunction was 74% lower than in the SCS arm. Discard rates were higher in the SCS group (24.1% *vs* 11.7%)[47]. However, there were no differences in biliary complications, ischemic cholangiopathy, incidence of PNF or graft and patient survival at 1 year[47]. A recently published study in discarded livers combined the use of DHOPE with subsequently controlled oxygenated rewarming and NMP[48]. From the 16 livers perfused according to the protocol, 11 were considered transplantable, which was decided based on pre-defined viability criteria. The authors conclude that the attributable percentage of transplantable livers in their center was increased 20% by using the DHOPE-COR-NMP protocol. This would have a major impact on the amount of transplantable livers if applied worldwide.

Pancreas: Machine perfusion of pancreas grafts is still in its infancy because of lower incidence of pancreas transplants. Besides, machine perfusion may increase edema of the pancreas due to its low-flow state. The use of machine perfusion in the pancreas is currently still in the pre-clinical experimental phase. Studies in the earlier years have favored SCS over HMP in preservation failure and post-transplant survival rates[49-51]. However, in more recent studies, results have been superior in machine perfusion[52]. No large data are yet available concerning the use of machine perfusion in pancreas transplantation.

Table 5 Currently ongoing clinical trials concerning *ex vivo* machine perfusion in liver transplantation

Name of study		Design	PI	*n*	Primary outcome	Intervention	Included donors	Results
DHOPE DCD	NCT02584283	RCT	Groningen	156	% NAS	2 h end-ischemic DHOPE	DCD III	October 2019
HOPE	NCT01317342	RCT	Zürich	170	Postoperative complications	1-2 h HOPE	DBD	July 2019
HOPE ECD-DBD	NCT03124641	RCT	Aachen	46	Peak ALT	1-2 h HOPE	ECD-DBD	June 2019
DHOPE-COR-NMP	NTR5972	Non-randomized	Groningen	16	Graft survival	DHOPE, gradually rewarming, NMP	Discarded livers (DCD and DBD)	11 livers transplanted 100% patient/graft survival, 9.1% ischemic cholangiopathy[48]
PIO	NCT03031067	Case control	Bologna	20	Graft function	2 h HOPE	ECD livers	February 2018
VITTAL	NCT02740608	Non-randomized	Birmingham	22	Patient survival	4 h NMP	Discarded livers (DCD and DBD)	March 2020
Liver WP2	ISRCTN39731134	RCT	Oxford COPE	220	Peak AST	Minimally 4 h NMP	All deceased donors	49.4% ↓ peak AST[47]
CORNET	ISRCTN94691167	RCT	Essen	40	Peak AST	1,5 h COR until 20 degrees (dual perfusion)	ECD	February 2021
DHOPE	NTR4493	Case control	Groningen	10	Graft survival 6 mo	At least 2 h of DHOPE	DCD III	↑ graft survival (P = 0.052) ↓peak ALT (P = 0.006) ↓bilirubin (P = 0.044)[46]
Unknown	NCT03837197	RCT	Bologna	260	Early allograft dysfunction	Minimally 1 hour of HOPE	ECD-DBD	December 2021

ALT: Alanine aminotransferase; AST: Aspartate transaminase; COR: Controlled oxygenated rewarming; COPE: Consortium for Organ Preservation in Europe; DBD: Donation after brain death; DCD: Donation after circulatory death; DHOPE: Dual hypothermic oxygenated perfusion; DCD: Donation after circulatory death; ECD: Expanded criteria donor; HOPE: Hypothermic oxygenated perfusion; NAS: Non-anastomotic strictures; NMP: Normothermic machine perfusion; PI: Principal investigator; RCT: Randomized controlled trial

Viability assessment

One of the benefits of machine perfusion is the possibility of viability assessment. However, rules concerning viability assessment are not set in stone. It still remains highly difficult, as often no highly predictive cut-offs of liver or kidney markers have been identified that could lead to either acceptance or rejection of the donor organ. Especially for HMP, viability assessment is largely unexplored.

Kidney: For NMP, Hosgood *et al*[8] developed a quality assessment score based on macroscopic perfusion, renal blood flow and urine output during NMP. The total amount of urine produced during NMP has proven to be significantly less in kidneys deemed unsuitable for transplantation[8]. It is unknown whether parameters during perfusion, such as flow and intrarenal resistance, may predict post-transplant outcomes.

Liver: For HOPE, fluometric analysis of released mitochondrial flavoproteins was shown to have a high predictive value of liver graft function after transplantation with an area under the curve of 0.926 for 90-day graft loss[53]. During NMP, liver viability can be assessed using a combination of transaminase release, glucose metabolism, lactate clearance and maintenance of acid-base balance[54]. Evaluation of bile pH may predict post-transplant biliary complications, such as ischemic cholangiopathy[54]. No correlation has been found for hepatic artery/portal vein resistance and hepatocellular damage[54]. Also, there was no difference in hepatic artery/portal vein resistance between non-transplanted livers and transplanted livers and transplanted and non-transplanted livers[48]. Liver enzymes, lactate and bile production has shown not to be sufficient for prediction of liver graft failure in the

recipient[54]. The following criteria have been described as being associated with successful transplantation of a normothermically perfused liver[54]: (1) Maximum bile pH > 7.5; (2) Bile glucose concentration ≤ 3 mmol/L or ≥ 10 mmol less than perfusate glucose; (3) Able to maintain perfusate pH > 7.2 without >30 mmol bicarbonate supplementation; (4) Falling glucose beyond 2 hours or perfusate glucose under 10 mmol/L which, on challenge with 2.5 g glucose, does subsequently fall; (5) Peak lactate fall ≥ 4.4 mmol/L per kilogram per hour; and (6) ALAT < 6000 iU/L at 2 h.

Current practice in the Netherlands

After publication of the results of the Machine Perfusion Trial in deceased donor kidneys, a committee was established in the Netherlands to implement this technique as standard of care. As a result, since January 2016, the Netherlands is the first country where HMP is standard of care for all deceased donor kidneys. Several studies, both experimental and clinical, are carried out in the Netherlands concerning the possible additional benefits of NMP in donor kidneys. In March 2018, the first kidney transplantation after NMP in the Netherlands was performed successfully in Erasmus Medical Center in Rotterdam. It was the start of a pilot study, the POSEIDON study, to assess the feasibility of kidney transplantation after NMP in the Eurotransplant Senior program. Because of the poor results of kidney transplantation in this program, the hypothesis was that those patients may benefit the most from an effort to improve organ quality by NMP[55]. The results from the NMP patients will be compared to a historical cohort of Eurotransplant Senior Program patients that have been treated with HMP. The study finished its inclusion, and the results are expected in the beginning of 2020. Furthermore, various experimental studies are currently carried out in discarded human kidneys and porcine kidneys concerning the best perfusion parameters to use when performing NMP. One of them is the PROPER study, a collaboration between Erasmus Medical Center, Leiden Medical Center and Groningen Medical Center, with the goal to improve discarded kidneys to make them transplantable. For liver *ex vivo* machine perfusion, the aforementioned DHOPE, DHOPE-DCD and DHOPE-COR-NMP studies are led by Groningen Medical Center. Various experimental studies on discarded livers or animal livers are currently carried out, and the results of those are about to follow.

CONCLUSION

Since the renewed interest in machine perfusion, major steps have been made by translating experimental research into clinical studies. For NRP, there is no evidence from RCTs yet. The currently available evidence suggests especially beneficial effects for improving outcomes of liver transplantation by reducing the incidence of biliary complications and ischemic cholangiopathy. For *ex vivo* machine perfusion in kidney transplantation, HMP has proven to be beneficial over SCS in an RCT, while NMP is currently under investigation. For *ex vivo* machine perfusion in liver transplantation, NMP has proven to reduce discard rates and early allograft dysfunction. Multiple RCTs, such as the DHOPE, are ongoing from which the results are awaited. In response to clinical studies, NRP and HMP for deceased donor kidneys have already been implemented as standard of care in the Netherlands.

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