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Machine Perfusion Plus for Extended Criteria Donor Liver Grafts: Making Every Liver Count

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ABSTRACT

Transplantation represents the most effective treatment for end-stage liver diseases but is limited by the shortage of healthy donor organs. Extended criteria donor (ECD) liver grafts are increasingly utilized in clinical practice to mitigate this challenge. However, impaired ischemic tolerance of these grafts jeopardizes organ viability during cold storage. Machine perfusion (MP) was designed to improve organ preservation and reduce posttransplant complications. Nevertheless, it is increasingly evident that MP alone may not preserve ECD grafts optimally. Increasing emphasis has thus been placed on modified MP strategies, including the use of different perfusates, modified perfusion modalities, and different therapeutic interventions. Here, we introduce a novel term, "MP Plus," denoting these additional strategies that are designed to restore organ function and potentially enable regeneration of ECD grafts. In this review, we summarize the existing and potential modified MP strategies and discuss their advantages in reconditioning different ECD grafts in clinical settings.

1. Introduction

Liver transplantation (LT) is currently the optimal treatment for end-stage liver disease, but organ shortages still limit the availability of this treatment to all patients who would potentially benefit [1]. In 2020, 12 609 patients were newly added to the liver transplant list, and the total number of candidates reached 24 936 in the United States, with only 8 906 liver transplants performed [2]. The use of extended criteria donor (ECD) liver grafts is increasingly pursued as a way to address this disparity [3]. The frequently described definition in the literature for ECD grafts generally includes elderly, steatotic, infected, and split liver grafts and grafts obtained from donors presenting circulatory death (DCD) [4]. Utilization of these organs is associated with an increased risk of posttransplant complications, including early allograft dysfunction (EAD), primary nonfunction (PNF), ischemic cholangiopathy, and death [5]. However, it is expected that, in addition to closing the gap between the numerous recipients on the waiting list and the available organs, careful selection with reconditioning of such grafts may also lead to improved posttransplant outcomes.

The organ discard rate was 8.4% in the United States in 2018, and this percentage could even be higher in other countries [2]. Machine perfusion (MP) is a sophisticated technique to mimic the physiological environment of a human body, attempting to maintain or enhance organ function [6]. With the increasing use of ECD grafts, there is an opportunity to extend the role of MP beyond assessment and logistics [7]. Recently, our team proposed a novel term, "MP Plus," to describe the use of MP combined with additional strategies aimed at reconditioning, repairing, and regenerating ECD grafts *ex vivo*. In this review, existing and potential newer "MP Plus" strategies are summarized, and their importance in different ECD liver graft settings is highlighted. The essential role of "MP Plus" in expanding the liver donor pool and further improving posttransplant outcomes is also discussed.

2. ECD liver grafts

ECD liver grafts are considered "marginal" due to various criteria and are labeled to confer an increased risk for poor graft and

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patient survival after transplantation. The definition of what constitutes an ECD is yet to be universally agreed upon [4,8]. However, some characteristics that are frequently described in the literature for ECD liver grafts include the following:

(1) Elderly liver grafts: The average age of liver transplant donors and recipients has increased markedly over time [9]. Despite a lack of a definitive classification of what constitutes an elderly liver graft, it is generally accepted that grafts over 70 years are frequently discarded. Elderly liver grafts often present fibrosis, steatosis, and viral infection, all conditions that are less tolerant to ischemic stress and reperfusion injury [10]. Although it has been reported that liver graft survival rates are not affected by donor age if well selected, there is potential to considerably expand the donor pool by utilizing marginal elderly liver grafts.

(2) Steatotic liver grafts: The prevalence of obesity is increasing worldwide and leads to a heavy burden of the associated metabolic syndrome and its hepatic manifestation. This has already impacted the deceased donor pool; therefore, strategies to increase the utilization of steatotic or fatty organs will become increasingly important in the near future [11]. Steatotic liver grafts are generally defined as grafts presenting $\geq$ 30% macrovesicular steatosis. Increased macrovesicular fat content is thought to exacerbate ischemia–infusion injury (IRI) through the release of reactive oxygen species, which are amplified by lipid peroxidation [12]. In addition, fat-loaded hepatocytes have been shown to cause compression of liver sinusoids, a situation ultimately leading to damaged hepatic microcirculation and an exacerbation of IRI [13].

(3) Donation after DCD: DCD is a modality of organ procurement in which organs are retrieved from donors presenting a circulatory arrest as opposed to donation after brain death, where the donor still has intact cardiopulmonary circulation. The use of DCD liver grafts varies from country to country due to legal constraints (e.g., DCD organs are forbidden by law in Germany) but is becoming more widely accepted and utilized as a potential source of liver grafts [14]. According to the European Liver Transplant Registry, DCD has gradually increased to represent almost 40% of adult postmortem LT in countries such as the Netherlands and Belgium [15]. The majority of DCD liver grafts originate from controlled DCD; the rapid retrieval technique is the preferred recovery strategy because it allows the shortening of both functional and true warm ischemia times [16]. DCD grafts are associated with a higher incidence of PNF, EAD, and ischemic cholangiopathy [17]. Therefore, advancements in technology aiming to extend the safe use of DCD organs should focus on minimizing ischemia times during organ preservation.

(4) Infected liver grafts: Viral infections such as hepatitis B and C virus (HBV/HCV) have previously been considered contraindications to transplantation. Active HBV infection refers to positive hepatitis B surface antigen (HBsAg), IgM anti- hepatitis B core antigen (HBcAg), and/or hepatitis B e antigen (HBeAg), and/or high HBV DNA levels [18]. Liver grafts from anti-HBcAg-positive donors can be safely used, especially in HBsAg-positive or anti-HBcAg/anti-HBsAg-positive recipients, on the condition that an adequate antiviral strategy is added postoperatively to avoid reinfection of the graft [19]. A study compared the outcomes of 42 HBsAg+ donors to 327 HBsAg– donors and found no difference in posttransplant complications or graft survival between the two groups [20]. Another study analyzed nine patients who received livers from anti-HBcAg-positive donors and revealed that livers from these donors should be prioritized for transplantation in a specific order: first to recipients who are positive for HBsAg, then to recipients who possess HBV antibodies, and finally to recipients who are HBV-naive [21]. By identifying the risk factors for HBV infection in both the donor and recipient prior to transplantation, it becomes possible to employ antiviral prophylaxis without discrimination, and this can potentially mitigate the scarcity of available donors.

For HCV, detecting viral RNA levels is the main diagnostic strategy [22]. Studies have indicated good short-term outcomes when combining direct-acting antiviral agents (DAAs) treatment and the use of HCV-positive livers in HCV-negative recipients. Doubt exists about the incidence of acute cellular rejection (reported in up to 16% of such recipients) [23]. Other viral (e.g., cytomegalovirus (CMV)), bacterial (donor infected status or sepsis), and fungal infections can also compromise post-LT outcomes.

(5) Split liver grafts: Split livers divide a full-size graft into two anatomically and functionally "smaller" grafts, a condition that may lead to organ dysfunction and impair outcome [24]. The use of split liver grafts has been demonstrated in some very specialized centers to be a good means to expand the number of available grafts. Due to the highly variable reported results and its inherent logistic problems, this procedure is unfortunately not performed on a large scale [25]. Therefore, split LT (SLT) is still regarded as a liver graft at risk [26].

Utilization of all forementioned ECD liver grafts has a potential risk of poor posttransplant outcomes; however, it is expected that their more widespread use will allow us to narrow the gap between the wait list and a few scarcities. In addition, it has been shown that in addition to their more frequent but careful selection, reconditioning could improve their safe use and may allow the generation of outcomes equivalent to those obtained after the use of standard criteria liver grafts.

3. MP and "MP Plus"

Rather than cooling the organ on ice to slow metabolic processes, MP aims to support normal metabolic functions as well as possible in a physiological environment and to provide a platform on which the organ can be evaluated, preserved, and even recovered [27]. MP devices have demonstrated the early success of bioengineering in improving posttransplant outcomes of different organs.

In 1812, a concept similar to "MP" first emerged in the monography of *Cesar Julien Jean Legallois*, which was muted as a replacement for the heart. It has been reported that the first closed system for delivering oxygenated blood was devised by Max von Frey and Max Gruber in 1885 [28]. Carl Jacobj created the "double hematizator" consisting of two blood oxygenation pumps and isolated lungs inserted between them in 1895 [29]. Alexis Carrel, the 1912 Nobel Prize winner, together with Charles Lindbergh investigated the rejuvenation of cultured tissues and first regenerated cells from the spleen, skin, pericardium, and portal vein of chick fetuses *ex vivo* [30]. These studies contributed primarily to the development of cardiopulmonary bypass but also formed a foundation for the pioneers of MP (Fig. 1).

In 1968, Belzer et al. [31] successfully transplanted the first kidney after 17 hours of hypothermic MP (HMP) using cryoprecipitate plasma. Brettschneider et al. [32] explored the use of HMP with diluted, heparinized, oxygenated blood through the portal vein and hepatic artery in mongrel donor dogs, and Starzl et al. [33] adopted this approach in 11 human LT. The first clinical trial comparing the feasibility and safety of HMP with static cold storage (SCS) in human LT involved 20 liver grafts and demonstrated that EAD rates were lower in the HMP group [34]. Based on a study of "orphan" livers, it was reported that the EAD rate, biliary

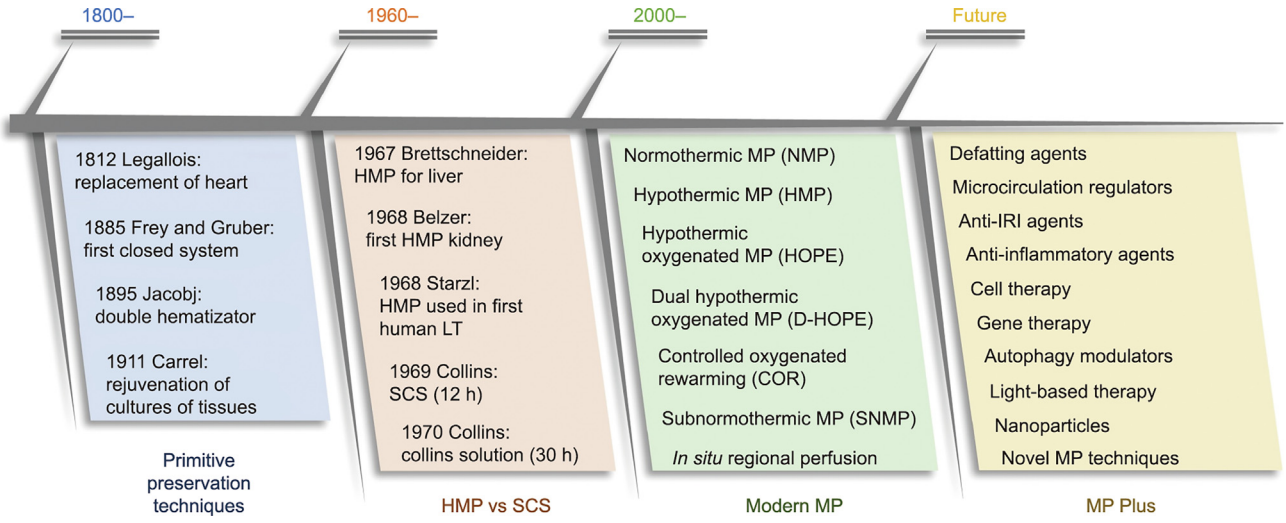

Fig. 1. Historical development of organ preservation, including MP. SCS: static cold storage.

complications, and hospital stay were all reduced in patients receiving HMP livers compared with SCS livers [35]. In 2021, a randomized controlled trial (RCT) conducted by van Rijn et al. [36] enrolled 160 patients, which also demonstrated that, compared to conventional SCS, HMP was able to reduce nonanastomotic biliary strictures in DCD livers. Based on a newly reported meta-analysis, including nine RCTs and 30 cohort studies, HMP was shown to significantly reduce the risk of nonanastomotic biliary stricture and EAD and to improve one-year graft survival in LT with ECD liver grafts [37].

The mechanisms through which HMP impacts liver grafts are yet to be fully characterized. HMP supplemented with oxygen delivered via the portal vein only (hypothermic oxygenated MP (HOPE)) or both the hepatic artery and portal vein (dual HOPE (D-HOPE)) has been investigated recently. Experimental data suggest that HOPE significantly reduces mitochondrial oxidative injury and further downstream tissue inflammation [38,39] The newly reported multicenter RCT conducted by Schlegel et al. [40] first investigated the impact of HOPE on cumulative complications within a 12-month period after LT and demonstrated that HOPE could decrease the risk of severe liver graft-related events. This conclusion is confirmed by a meta-analysis consisting of seven RCTs. HOPE reduced major complications, lowered the rate of "retransplantation," and allowed better graft survival than SCS did [41].

In 2018, van Rijn et al. [42] from the Netherlands reported a phase I clinical study including ten DCD livers preserved with D-HOPE after SCS. The degree of bile duct injury did not increase after reperfusion in the D-HOPE group, and there was less injury of deep peribiliary glands compared with controls. The same group has conducted an international multicenter phase III RCT enrolling 157 recipients comparing the efficacy of D-HOPE to that of SCS in relation to the prevention of nonanastomotic biliary strictures after transplantation using DCD liver grafts [43]. The trial inclusion has recently been completed, but the results are not yet published. However, another clinical report including 21 DCD grafts undergoing HOPE or D-HOPE reported that hospital stays were shorter, EAD rates were lower, and post-LT outcomes were better among patients receiving perfused liver grafts [44].

Limitations to hypothermic strategies include the limited functional assessment that can be made, the reduced ability to administer agents to modulate liver metabolism, and the limited benefits in minimizing ischemic times, and therefore logistics. Normothermic MP (NMP) is a technique allowing the perfusion of organs with oxygenated packed red blood cells in a colloid suspension at normal body temperature to mimic functioning in an optimized physiological state; this modality has been shown to reduce IRI clinically compared to that with SCS [45]. In addition, parameters monitored during NMP, including lactate clearance, pH maintenance, glucose metabolism, and bile production, were demonstrated to predict organ quality and posttransplant outcome [45,46]. Recently, the Meszaros et al. [47] reported that mitochondrial respiration with succinate and tissue viability remained stable during NMP and that marker of outer mitochondrial membrane damage, adenosine triphosphate (ATP) synthesis efficiency, and dissipative respiration could predict the clinical outcome after LT. Many clinical studies focusing on the comparative analysis of HMP, NMP, or SCS are now ongoing to shed light on the role of MP in LT.

Although NMP allows for biliary viability testing of ECD liver grafts prior to transplantation, it remains associated with IRI when applied after SCS. Considering that HOPE or D-HOPE could efficiently reduce IRI, researchers combined sequential D-HOPE and NMP to further improve the resuscitation and viability assessment of high-risk human donor livers. The first clinical trial of this combination demonstrated excellent results and a low rate of posttransplant cholangiopathy, thereby increasing the number of transplantable livers by 20% [48]. Most recently, excellent long-term outcomes have been reported by the same group from Groningen, who demonstrated that donor warm ischemia time could no longer be seen as a reason to decline a DCD liver for transplantation after sequential hypothermic and normothermic MP [49]. Therefore, the combination of different MP techniques may become a critical strategy to further improve organ preservation and resuscitation.

Due to its low cost and high convenience, SCS has been the mainstay of organ preservation for the past 40 years [50]. More recently, the use of MP has been revisited with renewed enthusiasm because of better bioengineering designs and improved cost-effectiveness. A wide array of strategies has now been explored for *ex vivo* MP [6], and a number of clinical trials have been investigating the efficacy of these strategies [51].

With the increasing use of ECD liver grafts, there is an opportunity to extend the role of MP, beyond assessment and logistics, to reconditioning and repairing allografts. A wide array of potential strategies has been investigated [37,40]. Here, we propose the "MP Plus" terminology, combining MP with strategies aimed at repairing, reconditioning, and optimizing ECD grafts prior to transplantation. The platform of "MP Plus" aims to maintain the organ

under a specific physiological environment by circulating a particular perfusate through the organ to ① prevent or attenuate organ injury resulting from *ex vivo* preservation and ② better preserve the organ and recover or even regenerate ECD grafts initially unsuitable for transplantation, thereby allowing expansion of the liver donor pool.

During the "MP Plus" process, a number of physical parameters are important because they affect organ quality: ① temperature; ② oxygenation; ③ filtration; ④ flow velocity; ⑤ perfusion pressure; ⑥ preservation of both arterial or venous circulation pathways and flow directions; and ⑦ liver movement [52]. To date, several companies and laboratories have developed *ex vivo* liver graft perfusion platforms, including the TransMedics Organ Care System (USA), OrganOx Metra (UK), XVIVO Organ Assist (Netherlands), and LifePort Machine (Switzerland) [6,27,52]. By designing a perfusion technology developed by a group of surgeons, biologists and engineers may enable the maintenance of injured human livers in a functional state for a long-time *ex vivo* and improve graft function [52].

At the 2020 International Liver Transplant Society (ILTS) MP Conference, it has been proposed that investigators should concentrate more on the prepublication of established study protocols and trials in ECD grafts and focus on clinical outcomes rather than on laboratory values as primary endpoints [53]. For ECD allografts, "MP Plus" may play a role beyond organ preservation, namely, identifying different pathophysiological processes and interfering with them to attenuate distinct situations. Examples of such strategies include defatting agents, microcirculation regulators, anti-IRI agents, anti-inflammatory agents, stem or progenitor cell therapies, gene and virus depletion therapies, and, last but not least, immunomodulation (Table 1 [54–87]). To achieve these goals, "MP Plus" will depend on the modification of the respective perfusates by the addition of beneficial agents or substances, elimination of harmful waste products, and optimization of bioengineering technologies when designing perfusion machines and delivery methods (Fig. 2).

4. "MP Plus" for ECD liver grafts

4.1. MP plus vascular therapy

Fat-laden hepatocytes are thought to cause compression of hepatic sinusoids with resultant damage to hepatic microcirculation. Animal models and human studies have both demonstrated that peribiliary vascular injury as a result of microthrombi is more prominent in DCD and may be the pathophysiological process that underpins the increased cholangiopathy associated with DCD grafts. These phenomena may also increase IRI [88,89]. NMP offers an opportunity to modulate and improve the microcirculation in grafts prior to transplantation. The parameters used for MP are intimately related to the microcirculation and vascular resistance changes during NMP and can be readily modified by a range of parameters, including temperature. Novel approaches to NMP, including hyperthermic MP (> 38 °C), have been proposed as a method to induce vasodilation, increase aerobic metabolism, and induce the production of protective molecules, such as heat shock proteins [54] (Table 1).

In addition to the modifiable parameters used for MP, exogenous vasodilators have also been proposed as a therapeutic strategy to improve steatotic liver function. The efficacy of improving graft function has been explored in the context of LT. Historically, some of these agents have been applied to SCS preservation solutions, but NMP now offers a novel approach to the administration of these agents. Examples include pentoxifylline (PTX) and carvedilol (CVD). PTX is a methylxanthine phosphodiesterase inhibitor that displays vasodilating properties in peripheral blood vessels, particularly in hepatic blood vessels [90]. Arnault et al. [55] added PTX to University of Wisconsin (UW) solution during SCS of isolated fatty rat livers; PTX significantly reduced vascular resistance and peliosis at the end of perfusion (Table 1). CVD is an adrenergic-blocking medication used to treat hypertension and cardiac ischemic disorders [91]. Ben Mosbah et al. [56] examined the effect of CVD in UW solution on the preservation of rat steatotic livers and demonstrated changes in vascular resistance and perfusion flow rates when livers were reperfused *ex vivo* (Table 1).

The presence of microthrombi has led authors to explore the use of thrombolytic therapies before naïvemplantation in DCD liver grafts [92]. Concerns exist about the use of thrombolytic therapies persisting or being transferred into the recipient; however, MP provides an opportunity to utilize the therapeutic benefit of such therapies while ensuring that agents have been metabolized or removed from the perfusate prior to transplantation and therefore minimizing any risk to the recipient. More recently, the efficacy of potent vasodilators has been examined in the setting of MP. Prostaglandin E1 (PGE1) combined with a short oxygenated warm perfusion of the liver graft from uncontrolled DCD resulted in improved liver function and decreased necrosis or apoptosis of hepatocytes [57,93] (Table 1). Epoprostenol is a prostacyclin analog and a potent vasodilator and inhibitor of platelet aggregation. In a porcine model of NMP, treatment with epoprostenol resulted in significantly lower serum levels of aspartate transaminase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), and higher bile production [58] (Table 1). Echeverri et al. [59] applied endothelin-1 antagonist BQ123, epoprostenol, and calcium channel antagonist verapamil in porcine DCD livers with NMP. Hepatic artery flow was significantly higher and AST levels were significantly lower in the BQ123 and verapamil groups than in the epoprostenol group (Table 1).

The complex pathophysiology observed in steatotic and DCD liver grafts may require an approach combining several of the abovementioned "therapeutic" strategies, such as vascular regulators, vasodilators, or thrombolytic therapies. Nitric oxide has also been shown to modulate hepatic microcirculation in a rat model of hepatic steatosis [94]. Nagai et al. [95] demonstrated its efficacy when insufflating grafts in combination with oxygenation via the suprahepatic vena cava during SCS. This strategy improved the microcirculation and portal venous flow after LT. Our group showed that surface conjugation of an anti-CD31 antibody enhanced the targeting of nanoparticles to graft endothelial cells of human kidneys undergoing NMP and successfully prevented IRI of endothelial cells [96]. Similarly, it has been suggested that these agents delivered during "MP Plus" of liver grafts could also exert a protective effect, and several groups are now working on this idea [97].

4.2. MP plus defatting therapy

Strategies to reduce intracellular hepatocyte triglycerides (TGs) have focused on either increasing the mobilization of intracellular TG stores and upregulating the oxidation of fatty acids (FAs) or upregulating cellular export. Cytoplasmic lipases are central to initiating the mobilization of intracellular TG stores and the conversion of TG into FA and glycerol. Adipose triglyceride lipase (ATGL) is considered to be the rate-limiting step in intracellular hepatocyte lipolysis; however, other potential pathways for upregulating FA catabolism exist. FAs have multiple intracellular functions, but one is as a ligand for nuclear receptors, upregulating the transcription of enzymes related to FA catabolism. Exploiting this function of fAs to increase their catabolism may be one such approach. Several drugs have been reported to reduce hepatic steatosis by elevating the level of intrahepatocellular lipid

Table 1
Main modalities and advantages of different "MP Plus" strategies.

ECD	MP	Plus	Advantages
MP plus vascular therapy			
DCD & steatosis	NMP	Hyperthermy [54]	Induce vasodilation, increase aerobic metabolism, induce protective molecules
Steatosis		Pentoxifylline [55], carvedilol [56]	Reduce vascular resistance, reduce peliosis at the end of perfusion
DCD		Prostaglandin E1 [57], epoprostenol [58], BQ123/verapamil [59]	Improve liver function, decrease hepatocytes death
MP plus defatting therapy			
Steatosis	NMP	GW7647, GW501516, hypericin, scoparone, forskolin, and visfatin [60]	A 65% decrease of hepatocyte triglyceride content after three hours
		Polyphenols, hypericin, scoparone, forskolin, and visfatin [61]	Defat steatotic rat livers with an optimized safety and reduced hepatotoxicity
		L-carnitine [62,63]	Reduce liver fat content both *in vitro* and in discarded liver grafts
		GDNF-loaded nanoparticles [64]	Reduction of fat in high-fat diet-fed mice livers
		Filters	Physically remove solubilized fats (not applicable yet)
MP plus anti-aging therapy			
Elderly	–	Irisin [65]	Improve autophagy (tested *in vitro*)
	NMP	Senolytics (dasatinib, quercetin, navitoclax, and HSP90 inhibitors) [66]	Remove the aggravating effects when elderly liver grafts undergo the process of transplantation
MP plus anti-infectious therapy			
Bacterial infections	NMP or HMP	Antibiotics [67,68]	Reduce bacterial counts, reduce endotoxin levels, improve organ function (applied in kidney and lung)
	Sub-NMP (33 °C)	Anti-inflammatory strategies (alprostadil, *n*-acetylcysteine, carbon monoxide, and sevoflurane) [69]	Decrease IL-6 and TNF-α, increase IL-10
	NMP	Antimicrobial agents [70]	Prolong the liver graft preservation time
		Antimicrobial agent (cefuroxime) [71]	Successful transplantation of septic donor livers
CMV infection	NMP	Immunotoxin (F49A-FTP) [72]	Reduce human CMV reactivation in recipients (applied in the lung)
HCV infection	NMP	Miravirsen [73]	Optimize liver function
	HMP	Methylene blue [74]	Reduce infectious HCV particles and transmission (applied in the kidney)
	NMP	Germicidal light or ultraviolet C irradiation [75]	Inactivate HCV in the perfusate (applied in the lung)
MP plus liver splitting			
Split liver grafts	HOPE	PEG35 and glutathione [76]	Reduce IRI and improve liver splitting
		D-HOPE [77]	Reduce the cold ischemia time, improve transplant logistics, prolong the preservation time
	NMP	Ferroptosis regulator (deferoxamine) [78]	Decrease intrahepatic iron, HO-1, HIFα, AST, and ALT
		Albumin, bicarbonate methylprednisolone, heparin, antibiotics, ursodeoxycholic acid, parenteral nutrition, lipids, and carnitine [79]	Long-term preservation of human hemi-livers, potential for liver regeneration *ex vivo*
MP plus cell therapy			
All ECD	NMP	MSCs [80]	Inhibit inflammatory reactions, alleviate rejection
DCD		MSCs [81]	Improve liver function, reduce hepatocyte apoptosis, repair mitochondrial damage
Discarded human livers		MAPCs [82]	Half of the grafts met the established criteria for organ viability
		Primary cholangiocyte organoids [83]	Repair bile duct injury
MP plus gene therapy			
All ECD	NMP or HMP	siRNA targeting Fas receptor and *p53* gene [84]	Reduce hepatocyte apoptosis
	HOPE	siRNA [85]	A proof-of-concept study
MP plus immunotherapy			
All ECD	NMP	Bioengineering filtration [86]	Remove passenger leukocytes
		MSC-derived extracellular vesicles [87]	Modulate the immune microenvironment

HSP90: heat-shock protein 90; GDNF: glial cell line-derived neurotrophic factor; MSC: mesenchymal stem cell; MAPC: multipotent adult progenitor cell; HO-1: heme oxygenase 1; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; PEGH35: polyethylene glycol 35; HIFα: hypoxia inducible factor α; AST: aspartate transaminase; ALT: alanine aminotransferase; siRNA: small interfering RNA.

catabolism [98]. Fig. 3 summarizes the mechanisms of intrahepatocellular lipid metabolism and agents that have been used for defatting, as well as the potential targets for future investigation.

Nagrath et al. [60] used isolated rat hepatocytes to demonstrate that a combination of the peroxisome proliferator-activated receptor α (PPARα) ligand GW7647, the PPARδ ligand GW501516, the pregnane X receptor (PXR) ligand hypericin, the constitutive androstane receptor (CAR) ligand scoparone, the glucagon mimetic and cyclic adenosine monophosphate (cAMP) activator forskolin, and the insulin-mimetic adipokine visfatin could be used to significantly reduce the intracellular fat content by 24% after 24 hours (Table 1). The authors then examined the efficacy of this combination by delivering these substances to *ex vivo* normothermically perfused steatotic livers and demonstrated a 65% decrease in the intrahepatocellular TG content after tthree hours of perfusion. Recently, Xu et al. [61] from Washington University developed a novel multidrug combination by replacing GW compounds with two polyphenols and successfully defatted steatotic rat livers via activation of the adenosine 5′-monophosphate-activated protein kinase (AMPK) pathway with optimized safety and reduced hepatotoxicity during NMP (Table 1). The efficacy of other agents in manipulating fat metabolism has also been explored. Rapamycin, a specific inhibitor of the kinase mammalian target of rapamycin (mTOR), has been shown to enhance FA oxidation, suppress lipogenesis, and induce TG secretion and macroautophagy [99,100]. Necrosulfonamide, an inhibitor of the mixed lineage kinase

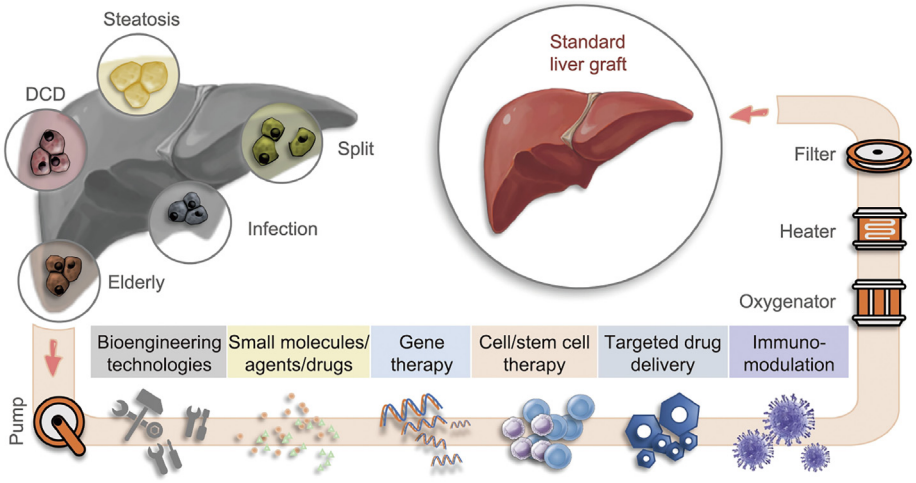

Fig. 2. Major types of ECD liver grafts and the potential role of "MP Plus."

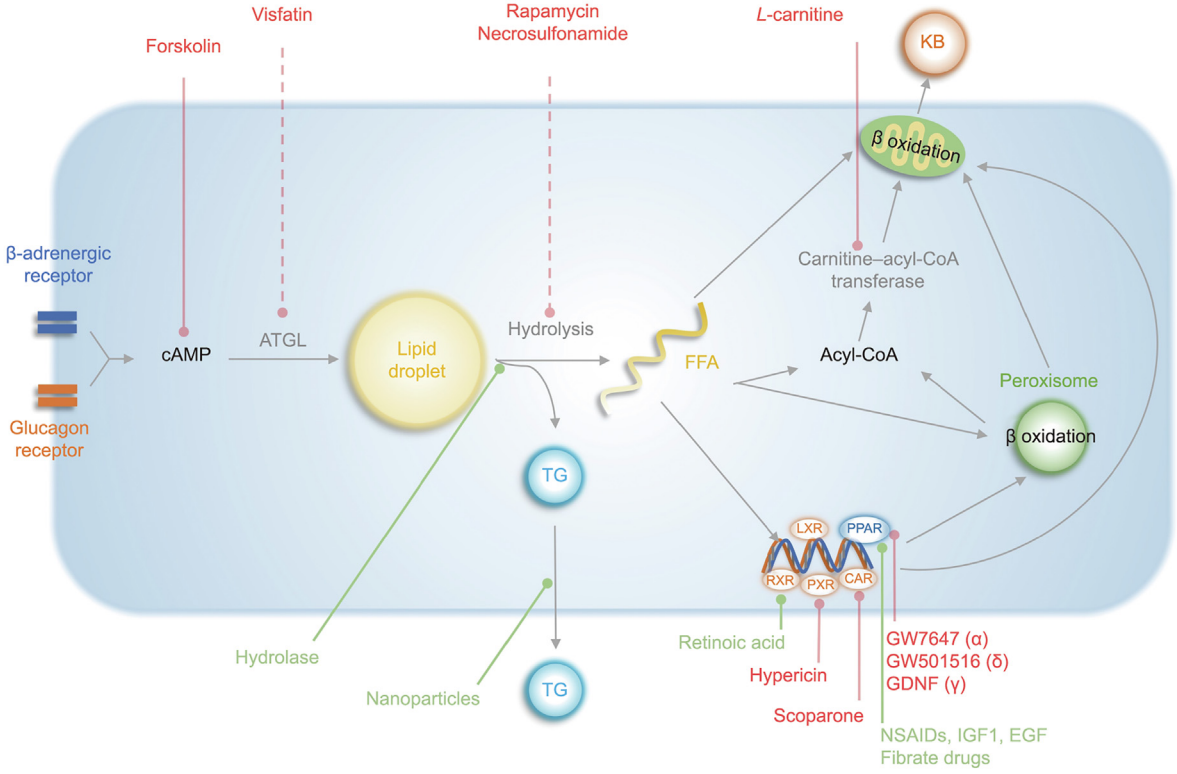

Fig. 3. Mechanisms of intrahepatocellular lipid metabolism and defatting agents. cAMP: cyclic adenosine monophosphate; FFA: free fatty acid; KB: ketone bodies; LXR: liver X receptor; RXR: retinoid X receptor; PXR: pregnane X receptor; PPAR: peroxisome proliferator-activated receptor; CAR: constitutive androstane receptor; NSAIDs: nonsteroidal anti-inflammatory drugs; IGF1: insulin-like growth factor 1; EGF: epidermal growth factor. Red solid line: directly functioning; red dotted line: indirectly functioning; green solid line: agents not studied yet. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

domain, regulates insulin sensitivity and TG secretion in the liver [101]. Aoudjehane et al. [102] combined rapamycin and necrosulfonamide with the agents previously reported by Nagrath et al. [60] to form a new defatting cocktail. This was efficacious in significantly reducing the hepatocyte TG content within 24 hours in primary human hepatocytes *in vitro*.

The augmentation of mitochondrial β-oxidation of FAs has led investigators to study the role of *L*-carnitine in defatting strategies. This amino acid has been demonstrated to successfully reduce liver fat content both *in vitro* and in discarded liver grafts with *ex vivo* NMP [62,63] (Table 1). The translation of the encouraging findings in animal models to human hepatocytes and livers is yet to be observed in several key areas and will require further investigation, including a more thorough characterization of the precise mechanism of action of the agents used in animal models. The use of filters to remove solubilized fats while on NMP would be appealing but is also yet to be demonstrated. Nevertheless, the spontaneous reversal of steatosis in the liver graft after transplantation (from 30%–60% to < 10% within 12 days) has been reported, demonstrating the potential for relatively rapid modification of the fat content

of livers *in vivo* [103,104]. Therefore, a prolonged preservation time is also important, which could provide a sufficient duration of time for any strategies of "MP Plus" to recondition organs *ex vivo*.

Manipulation of growth factors or expression of genes involved in lipogenesis and lipolysis might provide an alternative approach for reconditioning steatotic grafts. Overexpression of the growth factor glial cell line-derived neurotrophic factor (GDNF) in mice results in resistance to weight gain and hepatic steatosis induced by a high-fat diet (HFD) [105]. The overexpression of GDNF was associated with suppressed expression of PPARγ and increased expression of PPARα and β-adrenergic receptors, with an accompanying reduction in lipogenesis and increased lipolysis and lipid β oxidation [105–107]. Furthermore, exogenous administration of GDNF-loaded nanoparticles is protective against steatosis in wild-type mice fed a HFD. Efficacy has also been demonstrated in *ex vivo* perfusion models with a significant reduction in fat content in HFD mouse livers compared to controls [64] (Table 1).

4.3. MP plus anti-aging therapy

There is also some evidence to suggest a protective effect of NMP in elderly liver grafts, but whether this is simply related to minimizing cold ischemia rather than an alternative mechanism is unclear [108]. Therefore, excellent outcomes could also be achieved with elderly donors, and there is virtually no upper age limit. The challenge is how to optimize selection, procurement, and matching to guarantee better results with liver grafts from older donors.

One of the distinctive signs of older donor liver grafts is the process of autophagy. Wang et al. [109] investigated the role of autophagy in IRI via analysis of autophagy-related proteins (Agt4B) both *in vivo* and *in vitro* and confirmed that loss of Atg4B in the livers of old mice increases sensitivity to IRI, while increasing autophagy might ameliorate liver damage and restore mitochondrial function. Moreover, by applying transcriptomic profiling and protein analysis to evaluate temporal changes in gene expression during NMP, Ohman et al. [110] found that the activation of autophagy in discarded livers was associated with improved hepatocellular function. Hence, modulation of autophagy might be another therapeutic target for rehabilitating the function of "untransplantable" older livers. Irisin has been tested in aged hepatocytes and improves autophagy by increasing telomerase activity in hepatic IRI, which might be combined with MP to recondition elderly liver grafts [65] (Table 1).

Cellular senescence plays another important role in age-related and chronic liver disease, which may cause harm to both hepatocytes and cholangiocytes [111,112]. Cellular senescence in elderly liver grafts may limit the prognosis after LT, and therapies targeting senescence are attracting increasing interest. Senolytics are drugs that selectively target senescent cells and induce apoptosis, which could remove the aggravating effects when elderly liver grafts undergo transplantation [113]. Several senolytics, such as dasatinib, quercetin, navitoclax, and heat-shock protein 90 inhibitors are currently being investigated, and some are already being tested in clinical trials [66] (Table 1). During NMP, senolytics can be administered to the donor and directly target the isolated liver, which enables promising effects with a lower risk of side effects. However, no experimental or clinical research is investigating the combination of "MP Plus" with senolytics, and further studies are needed to clarify its efficacy.

4.4. MP plus anti-infectious therapy

Donor bacterial infection represents the most common risk of donor-derived disease transmission (approximately 30%), which was reported to cause nearly 30% of recipient deaths attributable to donor-derived bacterial infections [114]. Donor bacterial infection affects organ quality, especially infections by multidrug-resistant bacteria, which may cause severe sepsis and increase the hospital stay, morbidity, and mortality after transplantation [115]. However, recent reports demonstrated that LT from donor presenting, even multidrug-resistant *Acinetobacter baumannii*, bacterial infections could still achieve comparable outcomes [116,117]. Potential donors with positive bacterial infections should therefore not be excluded for organ transplantation. The combination of NMP or HMP with antibiotics has been shown in both rat kidney and human lung transplantation to significantly reduce bacterial counts and endotoxin levels and thus improve organ function [67,68] (Table 1). In a porcine transplantation study, liver grafts under NMP with anti-inflammatory strategies (alprostadil, *n*-acetylcysteine, carbon monoxide, sevoflurane, and subnormothermic temperature (33 °C)) significantly decreased the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α and increased the anti-inflammatory cytokine IL-10 after transplantation [69] (Table 1). Recently, Clavien et al. [70] reported their experience of treating successfully *ex situ* with antimicrobial agents during NMP for three days, a human liver from a donor presenting sepsis (Table 1). Similarly, another successful transplantation of septic donor liver was performed in Austria using the combination of NMP and an antimicrobial regimen (cefuroxime) [71] (Table 1). These case reports show that well-designed clinical studies of "MP Plus" in attenuating organ inflammation and recipient infection are needed to further confirm the role of MP in improving posttransplant outcomes.

Viral infections represent a major global public health problem and economic burden [118]. Despite the use of CMV antiviral prophylaxis for the high-risk CMV combination (seropositive donor to seronegative recipient), delayed-onset CMV infection or disease still occurs [119]. CMV has a seroprevalence in the adult population ranging from 30% to 100% according to age and geographical and socioeconomic factors [120]. The risk is greatest innaïvee recipients of organs from CMV-infected donors (D+/R− transplants), and seropositive recipients (D±/R+) have an intermediate risk [121]. The D−/R− pair has the lowest infectious risk. To date, only in human lung transplantation has it been shown that *ex vivo* perfusion with immunotoxin (F49A-FTP) significantly reduces CMV reactivation in recipients [72] (Table 1). This demonstrates again that "MP Plus" may provide a platform for targeting and killing latent CMV in a donor organ with promising results.

With the development of DAA antiviral agents, HCV-positive liver grafts have been increasingly utilized in LT [122]. Importantly, "MP Plus" also plays a critical role in modifying these grafts. Goldaracena et al. [73] from the Toronto General Hospital investigated the effect of miravirsen (a locked nucleic acid oligonucleotide that sequesters micro-RNA (miR)-122 and inhibits HCV replication) in a pig LT model under NMP, and drug uptake improved and optimized the outcome of LT (Table 1). Helfritz et al. [74] demonstrated that the combination of methylene blue with HMP reduced the load of HCV particles as well as their transmission during kidney transplantation (Table 1). Germicidal light or ultraviolet C irradiation during NMP has also been shown to successfully inactivate HCV in the perfusate in a short period in lung transplantation [75,123] (Table 1). All these novel approaches might be applied to treat HCV infection in liver grafts under MP. HBV is more complex than HCV, and complete clearance after infection is difficult to achieve with current therapies due to its integration in the DNA strain. To date, no study has focused on mitigating HBV infection of liver grafts under "MP Plus" [124].

4.5. MP plus liver splitting

HOPE has also been used in the preservation and repair of split liver grafts with encouraging results [125,126]. In 2022, the use of

a novel preservation solution with polyethylene glycol 35 (PEG35) and an increased concentration of glutathione combined with HOPE perfusion reduced the deleterious effects of IRI and improved the benefits of *ex vivo* liver splitting [76] (Table 1). Thorne et al. [127] showed that D-HOPE in a left lateral segment and extended right lobe liver split procedure reduced the cold ischemia time (CIT) and improved transplant logistics. Spada et al. [77] reported the first clinical case of D-HOPE in SLT, confirming the preclinical results on the feasibility of splitting livers during MP (Table 1). In addition to graft reconditioning, D-HOPE also allowed prolonged preservation time, an important aspect of the technique because it facilitated logistics for allocation and transplantation into two recipients. A prospective, pseudorandomized, dual-arm stage II clinical trial was designed to determine the safety and feasibility of prolonged D-HOPE (DHOPE-PRO) [128]. These investigations suggest that MP may be a valuable adjunct to expand the donor pool and improve the utilization of SLT in the future.

Zhang et al. [129] first compared NMP with SCS in preserving and transplanting split porcine livers. Based on emerging evidence that defines ferroptosis (iron-regulated hepatocellular death) as an IRI driver, Nazzal et al. [78] assessed whether the ferroptosis regulator deferoxamine in the NMP of split livers could modulate intrahepatic injury after *ex vivo* preservation (Table 1). Significantly decreased intrahepatic iron, heme oxygenase-1 (HO-1), hypoxia inducible factor α (HIFα), AST, and ALT were observed. This provides a preliminary proof of concept for the potential role of NMP plus ferroptosis regulators in reconditioning split liver grafts.

Recently, the use of NMP for the long-term preservation of 21 human hemilivers was investigated following studies demonstrating the protective effects of NMP on partial swine livers [79] (Table 1). Fourteen right and seven left hemilivers recovered from patients undergoing anatomic hepatectomies were perfused with a blood-based perfusate containing albumin, bicarbonates, methylprednisolone, heparin, antibiotics, ursodeoxycholic acid, parenteral nutrition, lipids, and carnitine. Ten of 21 hemi-livers were perfused *ex situ* with a standardized perfusion protocol for a week. Histology from biopsies after seven days of perfusion revealed no relevant necrosis or apoptosis. Furthermore, cellular proliferation was also demonstrated, indicating a regenerative capacity of partial human livers during prolonged *ex situ* perfusion. Moreover, a prolonged period of preservation, potentially augmented with the addition of growth factors or promoting genes to accelerate this process, provides an opportunity for significant regeneration *ex vivo* [97].

In the future, split liver grafts may constitute a larger proportion of the donor pool, and improved recondition and regeneration with "MP Plus" may help investigators realize this potentially underutilized donor pool.

4.6. MP plus cell therapy

Mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs) are immunomodulatory cells that have also been shown to have therapeutic effects on IRI [130,131]. MSCs derived from adipose tissues in a rat orthotopic LT model were shown to inhibit inflammatory reactions and significantly alleviate acute rejection following orthotopic LT [80]. Verstegen et al. [81] first reported *ex vivo* delivery of MSCs to liver grafts during MP, which could also inhibit inflammatory reactions and alleviate rejection (Table 1).

MSCs and MAPCs are two major types of cell therapies and offer a potential therapeutic strategy to recondition or recover marginal liver grafts prior to transplantation. Yang et al. [132] compared NMP plus MSCs with NMP and SCS in a rat DCD model. The addition of MSCs significantly improves liver function and liver histological damage reduced hepatocyte apoptosis and repaired hepatocyte mitochondrial damage. Potential mechanisms that have been proposed include the reduction of ferroptosis in hepatocytes [133] and the inhibition of the c-Jun N-terminal kinase–nuclear factor kappa-B (JNK–NF-κB) pathway reducing oxidative stress and promoting AMPK activation thereby reducing mitochondrial damage and increasing mitochondrial function [134]. Laing et al. [82] directly delivered MAPCs to six discarded human ECD livers under NMP (Table 1). After NMP plus MAPC delivery, half of the grafts met the established criteria for organ viability. Analysis of cytokines and chemokines in perfusates identified nine targets (IL-1b, IL-4, IL-5, IL-6, IL-8, IL-10, monocyte chemotactic protein-1 (MCP-1), granulocyte–macrophage colony stimulating factor (GM-CSF), and stromal cell derived factor-1a (SDF-1a)) related to the presence of MAPCs. Proteomic analysis revealed 259 unique proteins that have strong links to MAPCs and functional enrichment analysis demonstrated their immunomodulatory potential.

Another approach to mitigate biliary tract injury is the use of organoids. It has been demonstrated that primary cholangiocyte organoids can be engrafted into a discarded human liver during *ex situ* NMP and express key biliary markers (keratin 7 (KRT7), keratin 19 (KRT19), cystic fibrosis transmembrane conductance regulator (CFTR), and gamma glutamyl transpeptidase (GGT)) [83] (Table 1). Moreover, Roos et al. [135] recently reported the construction of human branching cholangiocyte organoids that could self-organize into complex tubular structures resembling the intrahepatic bile duct architecture. Although not yet applied during the process of LT, it can feasibly be combined with MP to further expand its utilization by reconditioning ECD liver grafts *ex vivo* and finally improving posttransplant outcomes.

The use of MP to enable the delivery of MSCs, MAPCs, or organoids to recover otherwise marginal or nontransplantable organs is appealing. Further work is needed to explore the therapeutic potential of other types of primary or stem cells, hematopoietic stem cells, and induced pluripotent stem cells for the regeneration of marginal grafts. Immune cell therapies (such as chimeric antigen receptor T-cell therapy and regulatory T-cell therapy) are also emerging as novel therapeutic strategies and can potentially be combined with MP in reconditioning or recovering ECD liver grafts [136].

4.7. MP plus gene therapy

Utilization of gene modulation agents in MP is also promising because of the targeted delivery to specific organs. Exogenous administration of small interfering RNA (siRNA) has been reported to attenuate the downstream effects of IRI and apoptosis. The first successful administration of siRNA during *ex vivo* MP in a rat model was performed by Gilloly et al. [137] under both normothermic and hypothermic conditions. By targeting the Fas receptor and *p53* gene, whose activation contributes to IRI through a proapoptotic pathway, the rat liver was successfully perfused in a stable state [84] (Table 1). Other targets, including V-rel reticuloendotheliosis viral oncogene homolog B (RelB), TNF-α, and proapoptotic caspases, have been demonstrated to have a significant effect on inducing IRI during LT, and silencing these genes prior to implantation might ameliorate IRI after blood reperfusion [138]. Recently, Bonaccorsi-Riani et al. [85] reported a proof-of-concept study that delivering siRNA compounds during HOPE can modulate organ function in a rat liver transplant model but needed a better design and appropriate doses of siRNA compounds (Table 1). As most ECD liver grafts are more susceptible to IRI, MP could offer great potential as an ideal delivery method for gene modulations. Many genes or molecules, studied *in vitro* or *in vivo*, have been shown to have a promising effect on reconditioning ECD liver grafts, and future studies are needed to improve the clinical applicability of "MP Plus" in mediating gene modulation *ex vivo*.

4.8. MP plus immunotherapy

During the process of infection, the intrahepatic inflammatory response and immunogenicity are altered. MP prior to transplantation makes it possible to modulate the immunogenicity of the graft, resulting in decreases in IRI and the innate inflammatory response, especially in ECD liver grafts. In a human LT clinical trial, HMP significantly reduced proinflammatory cytokine expression, relieving the downstream activation of adhesion molecules and migration of leukocytes, including neutrophils and macrophages, when compared to SCS controls [139]. In rat DCD liver grafts, Lauschke et al. [140] found that 24 hours of HMP significantly decreased human leukocyte antigen (HLA) class II antigen expression on postsinusoidal venular endothelium compared to SCS and improved the preservation of predamaged donor livers with higher immunogenicity.

Immunomodulation of grafts may shift the intrahepatic immune response from inflammatory to tolerogenic during MP, a condition that may reduce immune activation after transplantation of an infected liver graft. The combination of NMP and bioengineering filters successfully eliminates passenger leukocytes in the graft, leading to a significant reduction in recipient T-cell infiltration and, consequently, a decreased incidence of acute rejection [86] (Table 1). Furthermore, by adding MSC-derived extracellular vesicles in NMP, donor grafts exhibit milder posttransplant IRI but facilitate enhanced rehabilitation [87] (Table 1). These benefits have been achieved through the attenuation of immune cell activation and the preservation of endothelial barrier integrity, thus contributing to improved outcomes. Another promising approach to reduce inflammation and decrease the immune response during MP is the utilization of inhibitory RNAs, which target donor organ inflammation-related genes and decrease the immune response in recipients after LT [84]. All this information already shows that it will be possible to treat the organ during *ex vivo* perfusion to decrease immune activation and recondition immunologically infected liver grafts.

5. Conclusions

With an increasing global demand for LT, improving the utilization of ECD liver grafts is one approach to mitigate organ shortages. MP has potential roles in viability assessment, logistics, recovery, and reconditioning, especially of such organs at risk. During the last decade, there has been increasing interest in MP of donor livers, and considerable advances have been made in both experimental and clinical research in this area. Some of this work is moving beyond viability assessment and logistics toward the reconditioning of organs. If these strategies demonstrate efficacy, they will have a profound impact on the number of available grafts for transplantation and will also allow the introduction of a new potential pool of organs. MP will lead to a redefinition of what was previously considered to be an "untransplantable" allograft.

MP with all its different modalities, such as HMP, NMP, HOPE, D-HOPE, and sequential D-HOPE combined with NMP, has already been investigated thoroughly to achieve better organ preservation and assessment. "MP Plus," a term newly proposed in this review, denotes novel strategies combining different MP modalities and various allograft interventions and manipulations, all designed to restore organ function and to improve the regeneration of different kinds of ECD liver grafts. The wide variety of approaches, including vascular, defatting, anti-aging, anti-infectious, cell or gene therapies, as well as immunotherapy, have already been reported to highlight the opportunities and challenges in this fast-developing area of LT.

Continued investigations with translation from small and large animal models through declined human livers are warranted to ensure that these novel concepts and strategies will finally move from bench to bedside. Importantly, well-designed clinical studies, including randomized controlled trials (RCTs) with clearly formulated composite endpoints, must be conducted to obtain a higher level of clinical evidence. Such studies are also necessary to avoid an unjustified "override" of these costly ex situ procedures.

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Compliance with ethics guidelines

Zhoucheng Wang, Jack Martin, Jiongjie Yu, Kai Wang, Kourosh Saeb-Parsy, and Xiao Xu declare that they have no conflicts of interest or financial conflicts to disclose.

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