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Systematic review

SOME LIKE IT HOT. UTILITY AND MECHANISMS OF *EX-SITU* NORMOTHERMIC MACHINE PERFUSION OF THE LIVER

Damiano Patrono, Nicola De Stefano, Federica Rigo, Davide Cussa, Renato Romagnoli

Chirurgia Generale 2U, Azienda Ospedaliero Universitaria Città della Salute e della Scienza. Turin, Italy

Summary

The need for donor pool expansion in liver transplantation has increased utilization of grafts from so-called extended-criteria donors. Machine perfusion technology can improve preservation of these grafts, and its use has been associated with improved short- and long-term outcomes. During normothermic machine perfusion the graft is preserved at 37°C, and continuously supplied with oxygen and nutrients. The main advantages of this preservation technique are the reduction of ischemia-reperfusion injury, the prolongation of preservation time, and the possibility to assess graft viability or delivering therapies *ex-vivo*. However, it is still considered as a complex technique, which has elicited some reticence about its utilization among transplant professionals.

The aim of this narrative review is to give a synthetic but comprehensive update on normothermic machine perfusion in liver transplantation, discussing its fundamental principles and clinical implications. Technical and procedural aspects, along with physiological bases, will be discussed. Results of clinical trials will be summarized, including those highlighting the role of this technology in the delicate process of assessing liver viability and its impact on transplant logistics. Finally, latest findings in the field of basic and translational research on organ reconditioning will be reported.

Key words: normothermic machine perfusion, viability assessment, ischemic cholangiopathy, liver preservation, reconditioning

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Correspondence

Renato Romagnoli
Liver Transplant Unit, General Surgery 2U, Azienda Ospedaliero Universitaria Città della Salute e della Scienza di Torino, University of Turin, Corso Bramante 88-90, 10126 Turin, Italy
Tel.: +39 011 6334374. Fax: +39 011 6336770. E-mail: renato.romagnoli@unito.it

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INTRODUCTION

After the early experiences during the pioneer era of solid organ transplantation, the challenge of improving preservation and utilization of grafts from so-called extended-criteria donors (ECD) has brought machine perfusion (MP) to renewed life. In 2010, the study by Guarrera et al. [1] about the first series of liver transplants (LT) performed with grafts treated by hypothermic machine perfusion marked the beginning of a revolution in organ preservation in clinical liver transplantation.

Among the different techniques and approaches included under the broad term "machine perfusion", normothermic machine perfusion (NMP) is one of the modalities that has been most extensively studied and widely adopted. The founding principle of NMP is creating a physiologic environment in which

the liver is perfused at 37°C with an oxygenated perfusate and is supplied with nutrients, supporting its metabolism outside the body. This preservation modality avoids cold ischemia and allows reverting the detrimental effects of the initial period of static cold storage, if any. Thanks to the availability of transportable and fully automated devices, NMP can be initiated at the donor hospital, being referred to as normothermic machine preservation of upfront NMP. Otherwise, when NMP is started at the recipient hospital after an initial period of SCS, it is named end-ischemic or back-to-base NMP.

Despite the large number of clinical studies and the rapidly growing clinical experience, NMP is still perceived as complicated and potentially dangerous by many transplant professionals. Understanding the basic principles and molecular mechanisms of NMP, as well as acquiring a general knowledge about its clinical results and applications are fundamental prerequisites for any transplant surgeon willing to approach this fascinating yet demanding area of organ preservation.

Thus, this review aims at summarizing the physiology and mechanistic aspects underlying the clinical advantages offered by this preservation technique. This is relevant also in the light of the current debate about the pros and cons of different MP modalities, especially NMP and hypothermic oxygenated machine perfusion (HOPE). The implications of the possibility of testing graft function pre-LT and significantly extending preservation time will also be discussed, along with the perspectives offered by the utilization of NMP as a platform for delivering organ treatments and other potential applications.

MATERIALS AND METHODS

The PubMed database was accessed on May 18th, 2022, using the search terms "normothermic machine perfusion" AND "liver", with no time nor language restrictions, retrieving 254 articles. Literature review was performed by 4 Authors (DC, DP, FR and NDS) and any disagreement was resolved by consensus. Abstract were screened to select relevant articles, initially including 88 articles for full-text review. An additional 28 articles were included by cross-checking references from the included manuscripts, leading to the final inclusion of 116 articles. Of those, 41 (35.3%) were about clinical applications of NMP, 34 (29.3%) about NMP mechanism, and 41 (35.3%) about the use of NMP as a platform for organ treatment and other potential applications (Fig. 1).

Given the narrative nature of this review, a formal meta-analysis was not performed. Ethical approval was not sought.

RESULTS

Technical aspects

Although different NMP devices are available on the market, their basic elements are constant. These are the organ bowl, the perfusate reservoir, the oxygenator(s), the pump(s), the filter(s), the heat exchanger, and the tubing circuit. In addition, any MP device has several pressure, flow and temperature sensors, which are connected to a control system representing the "brain" of the machine.

The goal of NMP is recirculating through the organ a perfusate delivering oxygen and nutrients to sustain liver metabolism [2]. To meet the oxygen demand of tissues perfused at physiologic temperature, the presence of an oxygen carrier into the perfusate is required. Most frequently, third-party red blood cells are used, but these have been successfully replaced by a synthetic haemoglobin-based oxygen carrier [3,4]. Other components of perfusate used during NMP are colloids, nutrients (glucose, aminoacids, vitamins), insulin, heparin, vasodilators, bile salts and mineral supplements [5]. Perfusate composition may vary according to different protocols, especially depending on the duration of the perfusion. As NMP represents an ideal environment for bacterial growth, antibiotics and, less frequently, antifungals can be added to the perfusate.

To preserve liver viability during NMP, flow and pressure into the hepatic artery, portal vein and inferior vena cava must be maintained close to physiological levels [2,6]. This is normally accomplished by regulating the pressure generated by the pump(s) and/or by the mean of pinch valves placed at specific points of the tubing. At the same time, oxygen and carbon dioxide pressures must be regulated. Perfusate hyperoxygenation should be avoided, as it has been associated with persistent recipient vasoplegia after liver implantation [7]. NMP devices have incorporated the technology of modern membrane oxygenators, which allow the diffusion to blood (or perfusate) of gases through membranes with pores of diameter ≤ 1 micron, a size limit dictated by the need to avoid spillage of liquids through the membrane. By using a gas mixer, membrane oxygenators allow the independent control of the transference of O_2 and CO_2. As O_2 transference depends upon its percentage in the gas mixture pushed through the oxygenator, increasing O_2 fraction will determine an increase in blood or perfusate oxygen pressure. Conversely, being CO_2 more soluble, its removal depends upon the gas flow through the oxygenator. Thus, increasing gas flow will also increase CO_2 removal from the blood or perfusate [8]. Blood or perfusate flow thorough the oxygenator is also important for gas homeostasis. During extra-corporeal membrane oxygenation, a minimum 50-60 ml/kg/min of blood flow is necessary to ensure proper oxygenation, as increasing gas flow will have only minimal effect on blood

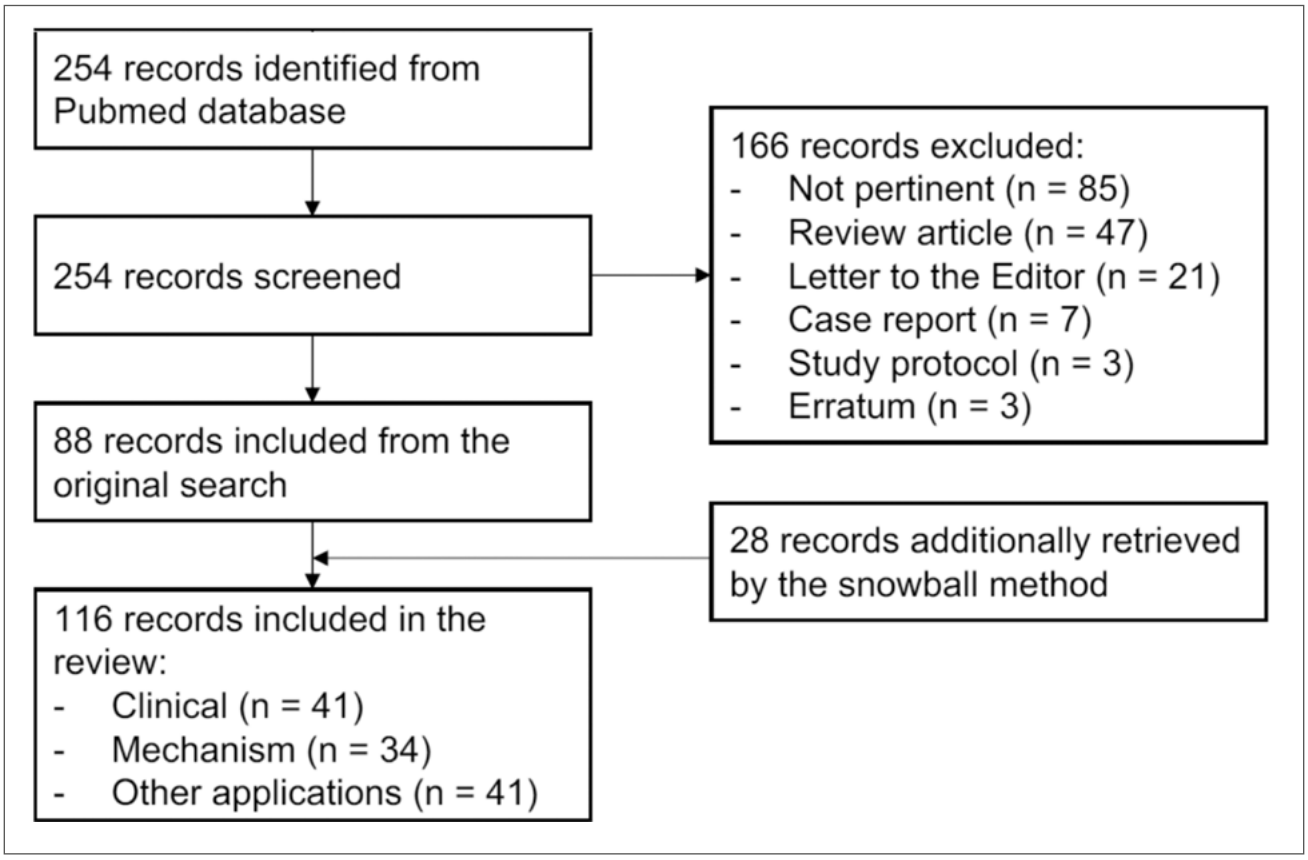

Figure 1. Literature search.

oxygenation. Conversely, due to its higher solubility, only 10-15 ml/kg/min of blood flow is necessary to effectively remove CO_2, which is mainly dependent upon gas flow [9]. Thus, it is evident how a tight regulation of vascular flows and pressure and of gas mixture and flow are necessary to achieve optimal perfusate oxygenation and effective CO_2 removal. Available commercial devices are characterized by different degrees of automation, which determines the amount of monitoring by trained personnel required to operate the device and also its transportability.

The complexity of NMP appears to increase proportionally to the duration of the perfusion, both in the experimental and clinical setting [10]. For example, to sustain bile production and counterbalance heparin degradation in the NMP circuit, continuous infusion of bile salts and heparin are needed for perfusions lasting up to 24 hours [11]. This concept is well reflected by the recent studies by the Zurich group about the development of a device allowing NMP to be extended up to several days. This fully automated prototype integrates different core technologies, including a dialysis unit, continuous infusions of insulin, glucagon and vasodilators, centrifugal pumps delivering a pulsatile flow into the hepatic artery and a continuous one into the portal vein, oxygenation, electrolytes and haematocrit control, and a liver case equipped with a soft inflatable mat mimicking diaphragmatic movements and avoiding pressure sores at the contact areas [12,13]. After obtaining authorization for its compassionate use, this device allowed successful transplantation of a very marginal liver preserved for 68 hours [14], opening the possibility of long-term viability assessment and reconditioning of grafts initially not meeting most commonly applied viability criteria [15-17].

Indeed, NMP can be applied at different timings during preservation. Normothermic machine preservation or upfront NMP is initiated at the donor hospital and has the advantage of reducing to a minimum the duration of initial cold ischemia time, which is nonetheless not completely avoidable due to the time necessary to complete the hepatectomy in the donor and prepare the liver for connection to the NMP device. According to Karangwa et al. [18] pre-NMP cold ischemia time should not exceed 3 hours to define NMP as upfront. By minimizing cold ischemia time, this approach should guarantee optimal preservation. Indeed, in the experimental setting it has been shown that even a short period of cold preservation has detrimental effect of graft recovery, especially in severely damaged grafts [19,20]. Not surprisingly, in both larger randomized

controlled trials having compared SCS with NMP [21,22], this last was initiated at the donor hospital. However, upfront NMP also poses significant logistical challenges. This approach requires a fully automated and transportable device that must travel to and from the donor hospital. The liver must be prepared and connected on the device on-site, which implies the availability of the necessary surgical instruments and of a suitable space in the donor theatre. The presence of aberrant hepatic arteries, even if their reconstruction can be accomplished once the liver is connected to the device [23], may further complicate this step. Once the liver is connected to the NMP device, a variable period of observation aimed at verifying haemostasis and equilibrating pH is necessary, which may prolong theatre occupation. Finally, any device malfunction or other problems arising during transportation may be particularly risky, as little or no intervention is possible during this phase.

For these reasons, many centres have preferred a so-called back-to-base approach, in which NMP is initiated once the organ arrives at the recipient hospital and, therefore, after a possibly long SCS time. Although this might be suboptimal from a preservation standpoint, this approach is undoubtedly simpler, and it is currently preferred by most centres [24-29]. Ceresa et al. [30] showed comparable outcomes in 31 patients transplanted with livers treated by NMP after a mean SCS time of 6 hours 1 minute, as compared to those of 104 patients receiving a graft treated by upfront NMP, suggesting that this approach might facilitate the adoption of NMP technology. These findings were in keeping with those from Bral et al. [31] showing a substantial comparability of outcomes between the two approaches. More importantly, end-ischemic NMP allows viability testing of grafts initially discarded for transplantation [32], which will be discussed more in details further on.

In both upfront and back-to-base approaches, however, NMP is started after a variable period of cold ischemia. To completely avoid cold ischemia, a third approach named "ischemia-free organ transplantation" (IFOT) has been described [33]. In IFOT, the liver is fully cannulated and connected to the NMP device *in-situ,* while still perfused by donor blood. Once the connections are established, systemic blood supply is halted by clamping inflow and outflow vessels, while NMP is simultaneously started. The liver is then procured and moved to the perfusion device. In the recipient, vascular anastomoses are performed under continuous NMP. Once the liver has been implanted, NMP is stopped while the vascular clamps are removed and the graft is reperfused by recipient blood [34]. As compared to conventional SCS, IFOT has been shown to prevent the activation of several pathways leading to ischemia-reperfusion injury (IRI) [35]. Clinically, this has resulted into better post-LT graft function (lower AST and ALT peak, lower end-of-transplant lactate level, lower incidence of postreperfusion syndrome, lower model for early allograft failure score) and improved outcomes (shorter ICU stay and reduced incidence and severity of non-anastomotic biliary strictures) (Dr. Zhiyong Guo – personal communication). However, IFOT is still prerogative of one centre and its reproducibility and advantages need confirmation.

Mechanism of action

Different hypotheses have been formulated to explain the observed advantages of NMP over SCS. The mechanisms behind the superior outcomes associated with the use of NMP in clinical LT are complex and multifaceted [36]. These mechanisms have been investigated at different levels.

Restoration of ATP production

Protracted ischemia and ATP depletion damage mitochondria and lead to reactive oxygen species (ROS) production once hepatocytes are abruptly reoxygenated, which in turn initiates the complex cascade of events finally leading to IRI. Furthermore, hypothermia slows down mitochondrial respiration by inhibiting the transition of complex I to its active form [37]. Overall, all dynamic preservation techniques have been shown to better protect mitochondria as compared to SCS [38]. During NMP, cellular metabolism is maintained by normal perfusate oxygenation, preventing or mitigating ischemia-related events and providing substrate for ATP production [39]. Simultaneously, anaerobic metabolism is reduced and this results in a decreased accumulation of selected metabolites, including lactate, and in the stimulation of pro-apoptotic pathways [40]. Xu et al. [41] demonstrated that porcine DCD livers exposed to 60 minutes of warm ischemia can be resuscitated by NMP due to the possibility of restoring tissue ATP content and improving mitochondrial integrity, which can already be observed after one hour of perfusion. As compared to sub-normothermic machine perfusion, NMP has been shown to reduce liver damage, especially during prolonged perfusions, by limiting shear stress on sinusoids, maintaining active metabolism, and satisfying oxygen demand [42]. Lonati et al. [43] demonstrated that NMP modulates the expression of several metabolites implicated in different cellular pathways, of which the majority are in the mitochondria.

An interesting approach to optimize metabolic and mitochondrial recovery, especially after an initial period of SCS, might be the sequential application of HOPE and NMP, a strategy that has been investigated by both the Groningen [4,28] and Birmingham [44] groups. This approach combines the beneficial effects of HOPE on mitochondrial respiration with the possibility of sustaining normal liver metabolism and testing viability during NMP. Boteon et al. [44] suggested that a combined protocol of HOPE + NMP

might attenuate oxidative stress, tissue inflammation, and improve metabolic recovery of highest-risk livers. Using this protocol, the same group reported the benefits of oxygen administration on liver microenvironment and ATP production [45]. They suggested that the use of NMP in combination with HOPE using a perfusate containing a haemoglobin-based oxygen carrier was associated with enhanced ATP synthesis, lower tissue expression of markers of oxidative tissue injury and reduced activation of inflammatory cells. This concept was clinically implemented by the team led by Prof. Porte in Groningen in the D-HOPE-COR-NMP trial [4], a prospective study of which the updated results have been recently published [28].

Modulation of inflammation

Downregulation of inflammatory pathways is pivotal to reduce IRI, which is characterized by a broad activation of inflammation as a response to the initial cellular damage. Cold-inducible RNA Biding Protein (CIRP), a group of proteins expressed as a response to hypoxia and hypothermia, are responsible for the activation of different inflammatory pathways [46]. The expression of these proteins appears to increase proportionally to hepatic IRI and conversely their reduction/blocking appears to be associated with reduced expression of inflammatory cytokines and reduced neutrophil infiltration, which results in reduced cell apoptosis and lower oxidative stress [47]. CIRP expression seems to be upregulated in grafts from DCD donors. In an animal DCD model, Liu et al. [48] observed increased CIRP levels during warm ischemia and demonstrated that NMP, as compared to SCS, reduced CIRP-mediated oxidative stress. Attard et al. [49] observed that the mitigation of the inflammatory response associated with end-ischemic NMP may promote endothelial regeneration and even prevent the shear stress derived from vessels cannulation, an effect that was particularly evident close to cannulation sites.

Modulation of the immune response

Modulation of the immune response is closely linked to the beneficial effects associated with NMP. In a much-needed study, Jassem et al. [50] investigated the differences in the activation of inflammatory pathways, apoptosis and IRI between DBD livers preserved by SCS *versus* NMP using microarrays, immunoprofiling of hepatic lymphocytes and immunochemistry staining. Changes in gene expression were evaluated on liver biopsies obtained at the end of preservation and 60 minutes after reperfusion into the recipient. Livers preserved by NMP showed a reduced expression of pro-inflammatory genes and an upregulated expression of regeneration pathways. More specifically, pathways linked to graft rejection, graft-versus-host disease, platelet/coagulation and immune response were downregulated in NMP livers. These changes were more apparent on liver biopsies obtained at the end of the preservation. Moreover, the characterization of hepatic mononuclear cells collected from the liver effluent at the end of preservation (representative of liver resident lymphocytes), showed that NMP liver contained less interleukin-17 and interferon-γ-producing lymphocytes and more regulatory T cells. At histological analysis of post-reperfusion biopsies, NMP livers showed lower degrees of apoptosis and necrosis and less neutrophil infiltration. Overall, these observations were interpreted as an overall lower activation of inflammation, enhanced regeneration, and modulation of the immune response in NMP livers. Gene and protein expression during NMP was also analysed by the Ohman et al. [51] in relation to liver function. In their study, human livers with adequate hepatocellular function during NMP were characterized by an early activation of innate immune response followed by activation of autophagy. Conversely, dysfunctional livers were characterized by delayed transcriptional activation of injury response pathways, suggesting that, while NMP activates repair mechanisms in response to IRI, its effectiveness might be related to perfusion duration and to the severity of the injury suffered before NMP. In a reduced-size pig LT model, Zang et al. [52] compared the expression of cytochrome C, caspase 3, Nf-KB p65 – transcription factor of several cytokines and chemokines in response to immune stimuli – and other inflammatory cytokines between pigs receiving a graft reduced during NMP *versus* SCS. Overall, levels of inflammatory cytokines (TNF-α, IL-1, IL-6) were lower after NMP, as was the expression of cytochrome C, caspase 3 and Nf-KB p65. These findings were consistent with a reduction of inflammation, mitochondrial injury, and apoptosis in the NMP group.

Other mechanisms

The effect of NMP on liver synthetic capacity has been investigated in several studies. Karangwa et al. [53] described the secretion patterns of prothrombin and plasminogen during NMP of discarded human livers, as indicators of the activation of coagulation and hyperfibrinolysis. In a previous study [54], the same group had suggested that end-ischemic NMP results in an activation of fibrinolysis, but not of coagulation, and that markers of fibrinolysis correlate significantly with hepatocellular function during NMP. They suggested that high perfusate D-dimers levels soon after the start of NMP can be considered a marker of severe IRI and a predictor of poor function. From a recipient perspective, Ionescu et al. [55] compared intraoperative thromboelastograms of patients transplanted with a liver preserved by NMP to those of matched recipients of a SCS liver. While there were no significant differences at pre-implantation thromboelastography, after implantation recipients of NMP livers had shorter R and R + K times, wider alpha angle and larger maximum amplitude

and G values. Hyperfibrinolysis was also mitigated by NMP treatment, in keeping with earlier and more effective recovery of synthetic function by NMP-preserved livers. Using a porcine DCD model, Gilbo et al. [56] evaluated the production of coagulation factors (FV, FVII, FVIII, FIX, FX) during NMP according to warm ischemia time (minimal *versus* 60-minutes warm ischemia time). The same data were obtained from perfusates collected during the COPE trial [22] and correlated to postoperative transaminase peak. In perfusate from life-sustaining livers that were utilized for LT, Authors observed that coagulation factors accumulated during NMP regardless of donor type or postoperative transaminase peak. However, in the experimental model, livers exposed to 60-minutes warm ischemia time had 2 to 6-fold lower coagulation factors levels, which were negatively correlated with perfusate AST and lactate, suggesting that a reduced synthetic capacity during NMP could represent a sign of severe injury, to be evaluated in further studies.

A further aspect favouring NMP over SCS relates to improved preservation of cholangiocytes and biliary tree. In a rat model of isolated-reperfused rat liver, NMP resulted in better preservation of the function and morphology of biliary epithelial cells, especially in DCD livers [57]. Biomarkers of bile duct injury (gamma-glutamyltransferase and lactate dehydrogenase in bile) were lower in NMP-preserved livers, whereas biliary bicarbonate concentration, reflecting biliary epithelial function, was higher. At histological examination, extrahepatic bile ducts of NMP-preserved livers demonstrated significantly decreased injury of the biliary epithelium, which was correlated to ATP depletion in SCS-preserved livers. This was confirmed also in a study from the Cleveland Clinic [58], in which 10 pig livers having been exposed to 60 minutes of warm ischemia were preserved via SCS or sanguineous NMP for 10 hours (5 per group), and then reperfused for 24 hours with whole blood in an isolated perfusion system to simulate transplantation. NMP was associated with better biliary epithelium preservation during the 24 hours of simulated graft reperfusion and promoted extrahepatic biliary epithelium and peribiliary glands regeneration.

Along with the production of coagulation factors, other aspects of liver metabolism appear to be maintained during NMP, as transaminase metabolism. In a study by the Toronto group [59], pig livers underwent NMP for 48 hours, during which a high transaminase solution was administered to attain a target perfusate level of 7500 IU/L. Authors observed that transaminases were progressively cleared from perfusate, indicating preserved liver metabolism. As liver function was not evidently affected by the increase in transaminase levels, authors argue that clearance of endogenous or exogenous transaminases during NMP could be used as a graft tolerance test and as a marker of graft function and viability.

Clinical results

The advantages of NMP over SCS have been evaluated by three randomized controlled trials [21,22,25] and some retrospective studies [24,60-63].

Most of the evidence about the superiority of NMP over SCS comes from studies in which an upfront approach was used. Ravikumar et al. [61] reported the first clinical series of 20 liver transplants performed using normothermic machine preservation, demonstrating the safety and feasibility of the technique. When recipients of livers preserved by NMP were compared with a 1:2 matched cohort of patients transplanted with a SCS-preserved graft, post-LT AST peak was significantly lower. This study paved the way for the pivotal COPE (Consortium for Organ Preservation in Europe) trial [22], which was a large multicentre trial comparing the outcomes of 121 patients receiving an NMP-preserved liver (DCD, n = 34, 28.1%) with those of 101 recipients of a liver preserved by standard cold storage (DCD, n = 21, 20.8%). The study met its primary endpoint and showed that NMP use was associated with a significantly lower AST peak (488 *vs* 965 IU/L, $p < 0.001$) in the 7 days following LT. Additionally, utilization rate was higher in the NMP group (88.3 *vs* 75.9%, $p = 0.008$), whereas the rates of postreperfusion syndrome (12.4 *vs* 33%, $p < 0.001$) and early allograft dysfunction (10.1 *vs* 29.9%, $p < 0.001$) were lower. This study, in which all patients had a magnetic resonance cholangiopancreatography performed 6 months after LT, failed to show a significant benefit of NMP on the development of anastomotic biliary complications or ischemic cholangiopathy. The incidence of ischemic cholangiopathy was lower in recipients of DCD grafts preserved by NMP, but without reaching statistical significance (11.1 *vs* 26.3%, $p = 0.18$). However, functional ischemia time was significantly longer in the NMP group (21 min *vs* 16 min, $p = 0.003$) and only one patient in each arm had clinically significant ischemic cholangiopathy requiring re-LT. The benefit of NMP on patient hemodynamics during LT was analyzed more in deep by Angelico et al. [60], who observed that after graft reperfusion patients receiving a liver preserved by NMP had higher mean arterial pressure, which was achieved with inferior vasopressor requirements and less blood transfusions. In contrast, a study from the Edmonton (Canada) group comparing outcomes of 9 livers preserved by NMP with those of a matched cohort of liver preserved by SCS failed to show any benefit of NMP [62]. In this study, ICU and hospital stay were significantly longer after NMP, and one NMP-preserved graft was discarded due to a technical error. More recently, the multicentre PROTECT trial compared the outcomes of 153 *vs* 146 LT performed using NMP or SCS, respectively [21]. This study confirmed COPE trial findings and met its primary endpoint, showing a significant reduction in the rate of early allograft dysfunction in NMP group (18 *vs* 31%, p = 001). Histological features of ischemia-reperfusion

injury were less evident after NMP. More importantly, this study demonstrated a significant benefit of NMP towards the development of ischemic cholangiopathy at 6 months (1.3 *vs* 8.5%, p = 0.02) and 12 months (2.6 *vs* 9.9%, p = 0.02). Less studies have compared end-ischemic NMP with SCS, probably because, in most cases, this approach has been aimed at recovering livers previously deemed unsuitable to be transplanted. In this setting, a comparison with SCS would be rather artificial. The only randomized controlled study having compared end-ischemic NMP with SCS was that by Ghinolfi et al. [25], which has also been the first machine perfusion study of this kind to be published. When applied to › 70-year-old donors (n = 10 in each arm), NMP use was not associated with significant clinical advantages over SCS, but electron microscopy suggested reduced ischemia-reperfusion injury in NMP-treated livers. Liu et al. [63] demonstrated the feasibility of end-ischemic NMP using an institutionally-developed device in a series of 21 LT (DCD, n = 8). When recipients of these livers were compared to a matched cohort of historical controls, transaminase peak and early allograft dysfunction rate were reduced. In a more recent retrospective study, Fodor et al. [24] compared the outcomes of 59 recipients of an NMP-treated liver (DCD, n = 9, 16%) with those of a matched cohort of LT performed using SCS, selected using 1:1 propensity score matching. While clinical outcomes were mostly comparable, recipient of NMP-treated livers developed less ischemic-type biliary lesions (3% *vs* 14%, p = 0.047).

Viability assessment and effects on organ utilization

One fundamental aspect of NMP utility is the possibility of testing *ex-situ* the function of livers to be transplanted. Many organs offered for LT, especially those from DCD donors, are discarded. Traditionally, liver acceptance has been based on the evaluation of donor factors [64] or donor-recipient matching [65-69], with the aim of modelling the risk of graft loss or recipient death associated with a specific case. However, graft acceptance varies widely in everyday practice, depending on centre attitude, experience, waiting list pressure and other logistical factors [70]. The ground-breaking potential of NMP lies in the possibility of assessing liver function and metabolism after the damage sustained during procurement and initial cold preservation in an unbiased environment, thus providing objective parameters guiding graft acceptance. Most widely adopted criteria for viability assessment during NMP are based on lactate and glucose metabolism, pH homeostasis, vascular flows, perfusate transaminases and bile production and composition [4,7,24,27-29,32,61,71-78]. However, at least in theory, any metabolic function can be tested during NMP and serve as a further element to assess liver viability [54,56,79]. A detailed description of the physiological bases of current viability criteria is beyond the scope of this review, as this will be the subject of another article in this issue. However, some concepts are worth stressing. First, when applied in the setting of livers initially deemed not acceptable for LT, NMP has allowed successful recovery and transplantation of 46 to 100% of livers, confirming its huge potential in expanding donor pool (Tab. I). However, primary non-function of NMP-treated livers has been anecdotally reported [29] and, more importantly, non-anastomotic biliary strictures have been observed [4,27,29,32]. This has led some centers to include in their protocols parameters to assess cholangiocyte viability [73], which has refined the ability to predict subsequent development of ischemic cholangiopathy [28]. However, these criteria have been criticized as they might be too restrictive [80]. The debate about how high-risk livers should be evaluated during NMP is still ongoing. Overall, viability assessment appears to be a science in its infancy, as reflected by the number of different protocols and by the substantial evolution of viability criteria over time at the same leading centres (Fig. 2) [4,28,32,72-74,76]. Furthermore, no protocol has been validated across different centres. At present, an element of subjectivity in the complex decision of accepting a liver graft still appears to be unavoidable [81].

Transplant logistics

Improving transplant logistics is one the main goals of machine perfusion. While this is not an exclusive feature of NMP [82,83], the possibility of significantly prolonging preservation time, with consequent obvious advantages on the logistics of LT, has been indicated as one of the most interesting properties of NMP since its early days [61]. Potentially, systematic use of NMP could transform LT into a semi-elective procedure, allowing avoiding out-of-hours procedures. From this point of view, the experience of the Innsbruck team might represent an organisational model to be taken as a reference [24,71]. The fundamental aspects of this model are extensive training, role separation, harmonic interaction between different figures (surgeons, anaesthetists, nurses and perfusionists), troubleshooting capacity and a holistic approach to NMP indications. At this centre, which applies NMP back-to-base upon organ arrival, the liver is handed over to the intensive care unit (ICU) team after it has been prepared and connected to the NMP device by the on call surgeon. In the ICU, the liver on the machine is treated and monitored like a patient. At the end of the preservation, device and perfusate parameters are reviewed and, if the liver is deemed viable and suitable, LT is started. This approach has allowed completely avoiding LT performed overnight. More importantly, the use of NMP is decided not only based on donor characteristics, but also taking into due consideration logistical aspects and recipient characteristics, an approach our group completely agrees upon [84].

The positive impact of NMP on transplant logistics has also been highlighted by the Birmingham group in the recently published NAPLES study [85]. In this study, outcomes of

Table I. Utilization rate after viability assessment by normothermic machine perfusion of livers having been initially discarded for transplantation

Author	n	DCD	Viability criteria	Time	Utilization rate	PNF	IC
Mergental et al., 2016 [74]	6	4/5 (80%)	Perfusate lactate level < 2.5 mmol/L or evidence of bile production + at least 2 of the following: 1) pH > 7.3; 2) stable vascular flows (hepatic artery flow > 150 ml/min and portal vein flow > 500 ml/min; 3) homogeneous perfusion and soft consistency	3 h	5/6 (83.3%)	0%	NA
Watson et al., 2017 [7]	12	9/12 (75%)	Changes in perfusate lactate, glucose and transaminases concentration + ability to maintain pH without supplemental bicarbonate	NA	NA	1/12 (8.3%)	(3/12) 25%
Watson et al., 2018 [29]	47	35/47 (74.5%)	Variables associated with successful transplantation: 1) Maximum bile pH > 7.5; 2) Bile glucose concentration ⩽ 3mmol/L or ⩾ 10 mmol less than perfusate glucose; 3) Ability to maintain perfusate pH > 7.2 with ⩽ 30 mmol bicarbonate supplementation; 4) Falling glucose beyond 2 h 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/kg/h; 6) Perfusate ALT < 6000 IU/L at 2 h	⩽ 6 h	22/47 (46.8%)	1/22 (4.5%)	4/22 (18.2%)
de Vries et al., 2019* [26]	7	7/7 (100%)	All of the following: 1) lactate <1.7 mmol/L; 2) perfusate pH 7.35 to 7.45; 3) bile production >10 mL; 4) biliary pH >7.45	2.5 h	5/7 (71.4%)	0%	0%
Matton et al., 2019 [73]	6	6/6 (100%)	1) Biliary bicarbonate > 18 mmol/L; 2) Biliary pH > 7.48; 3) Biliary glucose < 16 mmol/L; 4) Bile/perfusate glucose concentration ratio < 0.67; 5) Biliary LDH < 3689 IU/L	2.5 h	4/6 (66.7%)	0%	0%
van Leeuwen et al., 2020* [4]	16	16/16 (100%)	All of the following: 1) lactate <1.7 mmol/L; 2) perfusate pH 7.35 to 7.45; 3) bile production >10 mL; 4) biliary pH >7.45	2.5 h	11/16 (68.7%)	0%	1/11 (9.1%)
Mergental et al., 2020 [30]	31	14/31 (45.2%)	Perfusate lactate level < 2.5 mmol/L or evidence of bile production + at least 2 of the following: 1) pH > 7.3; 2) stable vascular flows (hepatic artery flow > 150 ml/min and portal vein flow > 500 ml/min; 3) homogeneous perfusion and soft consistency	4 h	22/31 (71%)	0%	4/22 (18.2%)
Reiling et al., 2020 [77]	10	5/10 (50%)	1) Lactate clearance to < 2 mmol/L within 2 hours; 2) Glucose metabolism as evidenced by a decreasing trend in serum glucose concentration by 4 hours; 3) Maintenance of physiological pH; 4) Stable hepatic arterial and portal venous flows; 5) Homogeneous graft perfusion with soft consistency of parenchyma 6) Bile production (no lower limit)	2-4 h	10/10 (100%)	0%	0%
Hann et al., 2021 [72]	5	0/5 (0%)	Perfusate lactate level < 2.5 mmol/L or evidence of bile production + at least 2 of the following: 1) pH > 7.3; 2) stable vascular flows (hepatic artery flow > 150 ml/min and portal vein flow > 500 ml/min; 3) homogeneous perfusion and soft consistency	6 h	NA	0%	NA
Quintini et al., 2022 [27]	21	13/21 (61.9%)	At least two of the following: 1) lowest perfusate lactate level < 4.5 mmol/L or a decrease of 60% from peak in the first 4 hours; 2) bile production rate higher than 2 mL/h; 3) stable HA flow of > 0.05 mL/min/g of liver weight and PV flow >0.4 mL/min/g of liver weight; 4) macroscopic homogenous perfusion and soft consistency	6 h	15/21 (71.5%)	0%	1/15 (6.7%)
van Leeuwen et al., 2022* [28]	54	53/54 (98.2%)	"Green zone" criteria**: 1) lactate < 1.7 mmol/L; 2) perfusate pH 7.35 to 7.45; 3) bile production > 10 mL; 4) biliary pH > 7.45; 5) Δ pH > 0.10; 6) Δ HCO3- > 5 mmol/L; 7) Δ glucose < -5 mmol/L	2.5 h	34/54 (63%)	0%	1/34 (2.9%)

* In these studies, normothermic machine perfusion was initiated after a period of dual hypothermic oxygenated machine perfusion and controlled oxygenated rewarming; ** Please see the original manuscript for a detailed description of viability criteria. Please note that the ultimate decision to utilize the liver for transplant was taken by the transplanting team, taking into due consideration the medical condition and urgency of the potential recipient.

Abbreviations: DCD: donation after circulatory death; PNF: primary non-function; IC: ischemic cholangiopathy; AST: aspartate aminotransferase; ALT: alanine aminotransferase

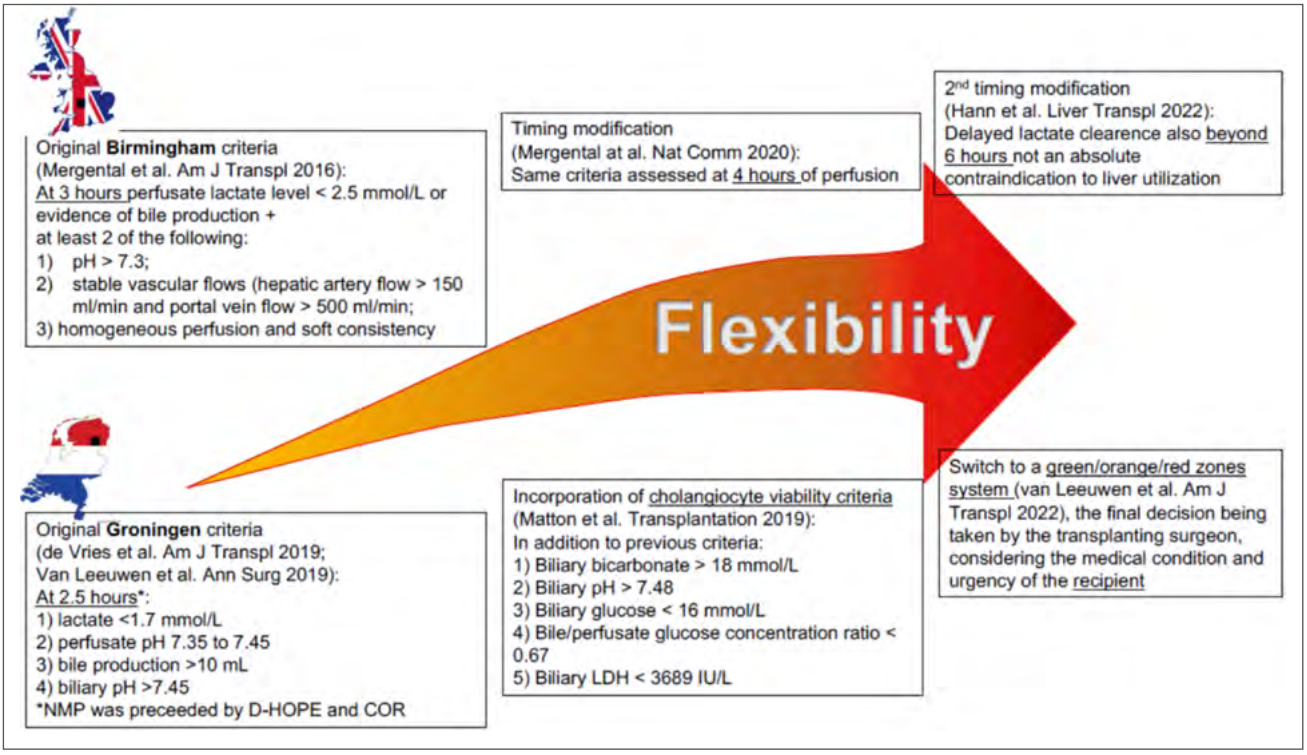

Figure 2. Evolution of viability assessment criteria at Birmingham and Groningen centers. .

repeat LT (re-LT) performed using NMP-preserved suboptimal liver grafts were compared to those performed with SCS-preserved optimal livers. As outcomes in both cohorts were comparable, authors conclude that NMP allowed achieving comparable outcomes despite the utilization of grafts from extended-criteria donors, thereby improving access to LT for patients awaiting re-LT. However, it is well possible that NMP also contributed to the good outcomes achieved in this study by buying more time to the transplanting surgeon having to perform a difficult recipient hepatectomy. By allowing longer preservation without the detrimental effects of prolonged cold ischemia time, NMP can relieve time pressure, which is of inestimable value especially in complex surgical cases, as re-LT frequently are. Finally, safely extending preservation time could facilitate organ sharing across longer distances, especially in the case of split livers. In-situ split requires an expertise that is not universally available in procurement teams, while ex-situ splitting might be not ideal, especially when the recipients of the two partial grafts are not located in the same hospital. Although livers considered for split procedures are highly selected, they can nonetheless suffer from the consequences of surgical manipulation and prolonged cold ischemia time and might benefit from an enhanced preservation modality. Liver splitting during NMP is technically feasible [86] and recently, prolonged NMP preservation of a split liver graft has been recently reported [87]. Ideally, the liver could be placed on the NMP device at the donor hospital, transported and split while on the device at the first recipient hospital, and then transported under continuous NMP at the second recipient hospital, an approach that would combine better preservation with the optimization of human resources. Obviously, the benefits and feasibility of this approach would be highly dependent on organizational and geographical factors, and several variations are possible.

Normothermic machine perfusion as a platform for organ reconditioning

As stated in the previous paragraphs, NMP has allowed achieving good LT outcomes with grafts from ECD and to recover approximately 70% of initially discarded grafts. In some cases, however, a graft can be considered too damaged to be utilized for transplantation, even following reconditioning and viability testing. NMP could be used not only to assess organ function, but also to improve it. Since during NMP the liver is metabolically active, it is potentially susceptible to treatments delivered *ex-situ*. Several strategies to improve organ quality have been explored in experimental studies, including defatting, gene silencing and cell-based therapies (Tab. II, Fig. 3).

Defatting strategies

Fatty livers, especially those with ⩾ 30% macrovesicular

steatosis, are associated with an increased incidence of short- and long-term complications following LT and are frequently rejected [88,89]. Pharmacological defatting during NMP could improve post-LT of fatty livers.

In the study by Nagrath et al. [90] the combination of forskolin (a glucagon mimetic), hypericin (a pregnane X receptor ligand), scoparone (a constitutive androstane receptor ligand), visfatin (an insulin mimetic), GW7647 (a PPARα ligand) and GW501516 (a PPARδ ligand) reduced triglyceride hepatocellular content by 65% in livers from obese Zucker rats after only 3 hours of NMP. Of note, when used at subnormothermic temperatures, the same cocktail failed to decrease liver lipid content, confirming the need of a fully active metabolism to successfully perform liver defatting [91]. In a study involving 10 discarded human livers randomly assigned to either NMP alone or defatting-NMP, addition of l-carnitine to the aforementioned cocktail further improved defatting [92]. In the treatment group, a 40% decrease in tissue triglyceride content and macrovesicular steatosis were observed after 6 hours of perfusion, along with improved mitochondrial function and a reduction in oxidative injury markers and inflammatory cytokines. Importantly, all livers treated with the defatting solution finally met transplant viability criteria, as confirmed by

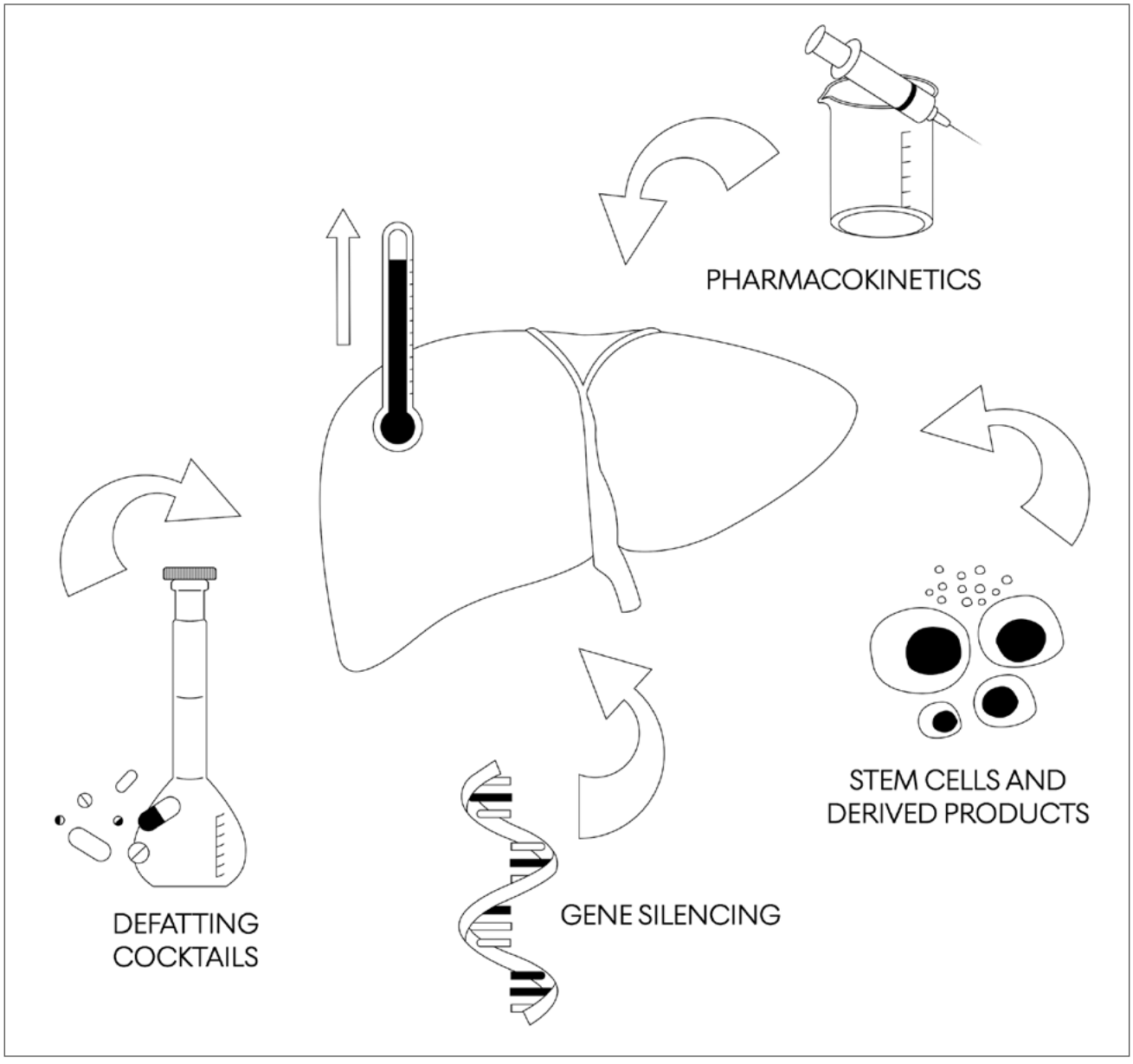

Figure 3. Organ treatment strategies during normothermic machine perfusion.

enhanced hemodynamics, lactate clearance and biliary function. More recently, in a rat model of 6-hours NMP, the same defatting solution downregulated pro-inflammatory genes (NF-κB and TNF-α), promoted the expression of fatty acid β-oxidation genes and increased liver viability, as confirmed by lower LDH release and better bile quality in the defatting group [93]. However, perfusate supplementation with defatting agents was associated with increased transaminase levels and insulin resistance.

Whether these protocols will be implemented in clinical practice is, however, a matter of concern, as some of these drugs still lack data about safety. The use of GW compounds is controversial since their administration has been associated with an increased risk of hepatic carcinogenesis [94]. Thus, glial cell line-derived neurotrophic factor (GDNF) has been proposed as an alternative [95]. In a murine model of defatting-NMP comparing GDNF to the forskolin/hypericin/scoparone/visfatin/GW-cocktail, the Authors demonstrated an equal effectiveness of the two treatments in terms of intracellular fat content reduction, but GDNF was associated with less LDH activity, a surrogate marker of hepatotoxicity. Similarly, the replacement of the GW compounds with epigallocatechin-3-gallate and resveratrol did not affect the ability of the solution to reduce tissue triglyceride content, but reduced hepatocyte injury and AKT phosphorylation, an indicator of the risk of malignant proliferation [94].

Finally, Banan et al. [96] perfused 2 discarded steatotic human livers adding only l-carnitine and exendin-4 to the perfusate. After 8 hours of NMP, a rise in triglyceride and LDL perfusate levels was observed together with a mild decrease (10%) in macrovesicular steatosis in one of the two cases.

However, in none of these studies treated grafts were eventually transplanted. Future experiments are required to clarify what should be the goal of liver defatting protocols and whether this will translate into superior clinical outcomes.

Gene silencing during NMP

RNA interference (RNAi) is a biological process that regulates the expression of protein-coding genes through a mechanism mediated by small complementary sequences of double-stranded RNA that can suppress the translation into proteins of specific mRNA. Small interfering RNA (siRNA), microRNA (miRNA) and short hairpin RNA (shRNA) are the central components of the RNAi system, and they exert their activity through specific post-transcriptional pathways [97]. RNAi has gained great interest as a possible source for new therapeutics in different clinical fields, including LT. Several studies have reported promising results with systemic injection of miRNA, shRNA or siRNA in rodent models of hepatic IRI [98].

However, despite its current success, some issues related to the use of RNAi still need to be resolved, such as *in vivo* instability and selectivity [97]. By providing a closed circuit in which the liver is isolated from other organs, machine perfusion clearly offers an ideal scenario for RNAi, as it guarantees organ-specific administration with no risk of systemic RNA degradation.

Goldaracena et al. [99] were the first to obtain RNAi during NMP. In their pioneering study, Miravirsen, an antisense miRNA122 oligonucleotide, was used during NMP of pig liver grafts to induce resistance to hepatitis C virus, demonstrating the possibility of *ex-vivo* gene silencing in the peri-transplant setting. In the study by Gillooly et al. [100], siRNA against Fas, a receptor involved in the apoptotic cascade, was successfully delivered to the perfused liver during NMP. To enhance cellular uptake, siRNA was coated with invivofectamine lipid nanoparticles.

However, while the possibility of graft reconditioning by RNAi is exciting, it is still at an early stage, since only a few preclinical studies have tested its feasibility. Further evidence about the effects on liver viability is warranted.

Cell-based therapies

Regenerative medicine uses stem cells and stem cell-derived products to promote the repair of injured tissues. In the context of solid organ transplantation, the regenerative and immuno-modulatory properties of stem cells make them an attractive source for graft reconditioning [101]. MP represents a unique tool to facilitate the application of regenerative treatments, as it avoids some of the side effects of the systemic stem cells injection while it facilitates their administration and studying their mechanisms of action. The combination of MP and stem cells or stem cells derivatives is still in its infancy, but some experimental studies have been recently published with promising results.

Mesenchymal stem cells (MSC) are multipotent stem cells that can be isolated from different sources, such as umbilical cord, bone marrow and adipose tissue. The group from Tianjin produced a quite large body of literature using bone marrow-derived MSC (BM-MSC) in the LT setting [102-109]. In a rat DCD model, combining BM-MSC with NMP resulted in improved liver function, better histology and less apoptosis as compared to SCS followed by or NMP alone [102-104]. BM-MSC treatment enhanced sinusoidal microcirculation by regulating ICAM-1, VCAM-1, vWF, ET-1 and eNOS expression, and reduced ROS production, Fe^{2+} imbalance and mitochondrial injury through the inhibition of the JNK-NF-kB pathway and the activation of AMPK. When applied in a rat model of DCD liver transplantation, the association of BM-MSC and NMP increased survival rate and reduced cholangiocyte injury, as confirmed by tissue histology and CK19 expression [105]. The same group showed that transfecting BM-MSC with heme oxygenase-1 (HO-1) gene improved their viability and produced even better results. HO-1 modified BM-MSC (HO-1/BM-MSC) added to NMP were superior not only to SCS and

Table II. Therapeutical strategies for organ reconditioning during normothermic machine perfusion.

	Author	Model	Treatment	Time	Mechanism	Outcome
Defatting strategies	Nagrath et al., 2009 [90]	Obese Rat	Forskolin, hypericin, scoparone, visfatin, GW7647, GW501516	3 h	↑ expression of lipid secretion signaling ↑ expression of fatty acid β-oxidation signaling	↓ tissue triglyceride content (-50%) ↑ VLDL and ketone secretion ↑ bile production ↑ oxygen consumption
	Boteon et al., 2019 [92]	Steatotic discarded human livers	Forskolin, hypericin, scoparone, visfatin, GW7647, GW501516, L-carnitine	12 h	↓ expression of inflammatory signaling ↑ expression of fatty acid β-oxidation signaling	↓ tissue triglyceride content (-38%) and macrovesicular steatosis (-40%) ↑ TG, cholesterol and ketone secretion ↑ bile production and quality ↑ ATP synthesis urea production ↓ vascular resistance and ALT
	Raigani et al., 2020 [93]	Obese rat	Forskolin, hypericin, scoparone, visfatin, GW7647, GW501516, L-carnitine	6 h	↓ expression of inflammatory signaling ↑ expression of fatty acid β-oxidation signaling	↑ TG and ketone secretion ↑ bile quality ↓ perfusate lactate ↑ hepatotoxicity and insulin resistance
	Taba Taba Vakili et al., 2016 [95]	High fat diet-fed mouse	GDNF	4 h	↑ lipolysis	↓ tissue triglyceride content (-40%) and macrovesicular steatosis (-50%) ↓ hepatotoxicity
	Xu et al., 2021 [94]	Obese rat	Forskolin, hypericin, scoparone, L-carnitine, epigallocatechin-3-gallate, resveratrol	4 h	↑ expression of AMPK signaling	↓ tissue triglyceride content ↓ hepatotoxicity
	Banan et al., 2016 [96]	Steatotic discarded human livers	Exendin-4, L-carnitine	8 h	NA	↓ tissue macrovesicular steatosis (-10% in 1 of the 2 treated livers) ↑ TG and LDL secretion
	Goldaracena et al., 2017 [99]	Pig	Miravirsen	12 h or 4 h + OLT	miRNA-122 silencing	Effective hepatic uptake *in vitro* suppression of HCV replication
	Gillooly et al., 2019 [100]	Rat	Fas siRNA	4 h	Fas gene silencing	Effective hepatic uptake

Table II. *continues.*

	Author	Model	Treatment	Time	Mechanism	Outcome
Cell-based therapies	Yang et al., 2020 [102]	30 min-DCD Rat	BM-MSC	8 h	↓ macrophage activation ↓ endothelial activation (↓ICAM-1, VCAM-1, vWF) ↑ microcirculation perfusion (↓ ET-1, ↑ eNOS)	↓ AST, ALT, ALP, lactate ↑ bile production ↓ Suzuki's scores, apoptotic cells ↓ mitochondrial damage
	Yang et al., 2020 [103]	30 min-DCD Rat	BM-MSC	8 h	↓ oxidative stress (↓MPO, MDA, ↑ GSH) ↓ expression of JNK-NF-kB signaling ↑ expression of AMPK signaling	↓ AST, ALT, lactate ↑ bile production ↓ Suzuki's scores, apoptotic cells ↓ mitochondrial damage
	Sun et al., 2021 [104]	30 min-DCD Rat	BM-MSC	6 h	↓ oxidative stress and ferroptosis (↓ MDA and PTGS2, ↑ GSH and GPX4) ↓ autophagy (↑ FTH1 and P62)	↓ AST, ALT ↑ bile production ↓ Suzuki's scores
	Hou et al., 2019 [105]	30 min-DCD Rat	BM-MSC	4 h	NA	↑ post-transplant survival ↓ Biliary injury (↑ CK19+ cells)
	Cao et al., 2020 [106]	30 min-DCD Rat	HO-1/BM-MSC	4 h	↓ IL-1β, IL-6, TNFα, HMGB1 ↓ expression of JNK-NF-kB signaling	↑ post-transplant survival ↓ AST, ALT, ALP, gGT ↓ Biliary injury (↑ CK19+ cells) ↓ Suzuki's scores
	Cao et al., 2021 [107]	30 min-DCD Rat	HO-1/BM-MSC	4 h	↓ NK-T and T-CD8+ infiltration ↓ IFN-γ, TNF-α, IL-2	↑ post-transplant survival ↓ acute cellular rejection ↓ AST, ALT, ALP, gGT, bilirubin ↓ apoptotic cells
	Wu et al., 2022 [108]	30 min-DCD Rat	HO-1/BM-MSC	4 h	↓ Dendritic cells maturation ↓ T-CD4+ infiltration ↓ IFN-γ, TNF-α, CCL-2, CXCL-9, CXCL-10	↑ post-transplant survival ↓ acute cellular rejection ↓ AST, ALT, ALP, bilirubin ↓ apoptotic cells
	Tian et al., 2021 [109]	30 min-DCD Rat	HO-1/BM-MSC	4 h	↑ expression of Wnt signaling	↑ post-transplant survival ↓ AST ALT, ALP, gGT, bilirubin ↓ bile ducts integrity ↓ apoptosis and ↑ proliferation of peribiliary glands cells
	Verstegen et al., 2020 [110]	15-45 min-DCD Pig	BM-MSC	30min HMP -> 4 h NMP	↑ IL-6, IL-8	Effective hepatic uptake Immunomodulatory effects
	Laing et al., 2020 [111]	Discarded human livers	MAPC	6 h	↑ IL-4, IL-5, IL-6, IL-8, 10, MCP-1, SDF-1α, IL-1β, GM- CSF	Effective hepatic uptake Immunomodulatory effects

	Rigo et al., 2018 [119]	Rat	HLSC-EV	4 h (hypoxic NMP)	↓ HIF-1α, TGF-1	↓ AST, LDH ↓ Suzuki's scores, apoptotic cells
	De Stefano et al., 2021 [120]	Rat	HLSC-EV	6 h	NA	↓ AST, ALT, phosphates, vascular resistance ↓ total HCO_3^- need ↑ bile production ↓ necrosis ↑ proliferation
	Sampaziotis et al., 2021 [121]	Discarded human livers	Gallbladder cholangiocyte organoids	100 h	Differentiation of gallbladder organoids into intrahepatic cholangiocytes	~40-85% regeneration of injected intrahepatic ducts ↑ bile production and quality
Other drugs and pharmacokinetics	Beal et al., 2019 [122]	Rat	Enkephalin	4 h (hypoxic NMP)	↓ oxidative stress (↓MDA, ↑Glutathione) ↓ expression of p38 and JNK signaling ↑ expression of p-Akt, PI3K, and Bcl-2 signaling	↓ ALT ↑ ATP ↓ apoptotic cells
	Westerkamp et al., 2020 [123]	Rat	Metformin preconditioning	3 h	NA	↑ ATP ↑ bile production and quality ↓ lactate and glucose ↓ post-transplant AST
	Haque et al., 2021 [124]	DCD discarded human liver	Plasminogen + tPA	12 h	NA	↓ peribiliary vascular plexus injury scores No intramural bleeding
	Garcia-Aroz et al., 2022 [125]	30-60 min-DCD Pig	CD47 monoclonal antibody	6 h	↓ neutrophil infiltration ↓ expression of TSP-1/CD47 signaling ↑ expression of pERK signaling	↓ AST, ALT ↑ bile production
	Del Turco et al., 2022 [126]	Discarded human livers	Cerium oxide nanoparticles	4 h	↓ oxidative stress (↑ GSH, SOD and CAT)	↓ mitochondrial damage ↓ lipid peroxidation products
	Stevens et al., 2021 [127]	Pig	atorvastatin, pitavastatin, rosuvastatin	7 h	NA	Toxicity, pharmacokinetics and drug-to-drug interaction analyses
	Tingle et al., 2022 [128]	Steatotic discarded human livers	2,4-dinitrophenol	25 h	NA	Toxicity and pharmacokinetics analyses ↑ oxygen consumption ↑ bile quality ↓ necrosis

NMP alone, but also to NMP + BM-MSC in improving rat liver function and recipient survival [106-109]. Two weeks after transplantation, serum levels of IL-1β, IL-6, TNF-α, HMGB1 and TLR4/NF-κB pathway molecules, all key actors of the inflammatory response to IRI, were significantly lower in the HO-1/BM-MSC group [106]. When applied to an acute rejection murine liver transplantation model, HO-1/BM-MSC significantly reduced tissue injury and apoptosis, being equal to calcineurin inhibitors in protecting from acute cellular rejection [107,108]. In particular, HO-1/BM-MSC reduced INF-γ expression and NK and CD8+ T cells infiltration into liver grafts [107], and limited dendritic cells and CD4+ T cells activation [108]. Finally, the HO-1/BM-MSC treatment was also effective in reducing bile duct injury after transplantation [109]. The Authors identified in the activation of the Wnt signaling pathway the mechanism by which HO-1/BM-MSC promoted the proliferation of the residual peribiliary glands cholangiocytes.

The infusion of MSC and Multipotent Adult Progenitor Cells during machine perfusion of porcine [110] and discarded human livers [111] proved the feasibility of this technique also on larger-size liver grafts, and confirmed the ability of stem cells to modulate key inflammatory genes *ex-vivo*.

Human liver stem cells (HLSC) are a mesenchymal-like stem cell population with regenerative and hepatoprotective activity [112-114]. Extracellular vesicles from HLSC (HLSC-EV) play a central role in the paracrine mechanism of action of HLSC and have been shown to be effective in several models of acute and chronic liver injury [115-117]. Compared to stem cell-based treatments, HLSC-EV could be advantageous in terms of genetical stability, storage conditions and administration route, especially in the transplantation setting [118]. Our group firstly reported the successful administration of HLSC-EV during hypoxic-NMP [119]. After 4 hours of NMP the hepatocyte uptake of HLSC-EV was confirmed by epifluorescence microscopy and treated livers showed reduced cytolysis, tissue injury, and overexpression of HIF-1α and TGF-β1. Furthermore, to investigate the effects of HLSC-EV in a high-risk DCD condition, we developed a prolonged warm ischemia model of rat liver NMP enriched with HLSC-EV [120]. The organs treated with HLSC-EV showed less transaminases release and preserved liver function, with enhanced pH self-regulation and phosphate utilization. Interestingly, when higher doses of HLSC-EV were added to the perfusate, a further improvement was observed on bile production, hemodynamics, tissue necrosis and cell proliferation, suggesting a dose-response correlation.

Another fascinating treatment is represented by organoids, three dimensional multicellular structures that mimic their corresponding *in vivo* organ and can be used to repair injured tissues [101]. In a landmark study, Sampaziotis et al. [121] injected cholangiocyte organoids into intrahepatic bile ducts of human donor livers and showed that after 100 hours of NMP the treatment fully protected the biliary tree from ischemic cholangiopathy and regenerated up to 85% of the injected ducts.

Taken together, these preliminary studies have paved the way for the administration of cell-based therapies during NMP, but further research is required to investigate their safety and efficacy *in vivo*.

Further applications of NMP in drug delivery

Enkephalin, an opioid agonist of delta receptors, has been administered during rat liver NMP obtaining a reduction of oxidative stress, as demonstrated by lower AST, MDA and glutathione levels, increased ATP synthesis and preserved tissue integrity [122]. The Groningen group has used metformin to precondition rat livers before NMP, resulting in improved hepatobiliary function and lower post-transplant transaminases [123].

Administration of thrombolytic agents has been proposed as a possible strategy to reduce ischemic cholangiopathy in DCD livers, but it is associated with an increased risk of intraoperative bleeding. Haque et al. [124] treated discarded DCD livers with tissue plasminogen activator (tPA) during NMP. *Ex-vivo* tPA administration reduced peribiliary vascular plexus injury while avoiding intrahepatic bleeding.

More recently, humanized antiCD47 monoclonal antibodies have been infused into porcine livers before NMP to block the endothelial CD47 cascade and reduce IRI [125]. CD47 signaling pathway blockade increased bile production and reduced transaminases levels and neutrophil infiltration in livers suffering from up to 60 minutes of warm ischemia.

In another recent study involving 9 discarded human livers, Del Turco et al. [126] successfully administered Cerium oxide nanoparticles during NMP, showing reduced oxidative stress and preserved mitochondrial morphology.

Finally, NMP can be used not only to deliver therapies aimed to ameliorate organs for transplantation, but also to study their pharmacological properties. In fact, perfusate, tissue and bile samples can be easily collected during NMP to perform toxicity analyses, pharmacokinetics profiling and/or pharmacodynamics assessment [127,128].

DISCUSSION

Technological evolution has allowed NMP of the liver to become reality. The main advantages of NMP over SCS are improved graft preservation together with the possibility to prolong preservation time and to assess liver viability [39]. Its wider implementation could potentially transform LT practice, radically modifying transplant logistics and possibly allowing a safe expansion of donor pool. The recent technical implementations, which have allowed achieving the previously unimaginable goal of

perfusing a liver for a week [12], unfold new scenarios for long-term liver evaluation, reconditioning and repair.

Given the complexity of NMP, a thorough understanding of its technical and mechanistic principles is necessary for anyone willing to approach this fascinating but demanding area of organ preservation. Appropriate training and technical support appear of paramount importance, given that surgical errors or device malfunction can rapidly result in organ loss during NMP [62]. Troubleshooting procedures and a back-up plan for rapid conversion to SCS should be well established before embarking in this delicate organ preservation modality. Furthermore, a thorough understanding of NMP physiology is necessary to correctly interpret different viability parameters and to put them together in a coherent picture. Current viability criteria should still be considered as centre- and setting-specific, possibly evolving in the near future, and an adjunct, rather than a substitute, of critical decision-making. Finally, clinical NMP implementation may require a rethinking of organizational models and the availability of new professional figures (i.e. organ perfusionists), with a clear definition of procedures and roles [71].

In conclusion, we are at the beginning of a new era in organ preservation and NMP represents one promising preservation strategy, with a potentially disruptive impact on how LT is conceived. The future will tell us to what extent the many potentialities of NMP will found application in clinical practice.

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Conflict of interest statement

The Authors declare no conflict of interest.

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Authors' contributions

DP: study design, literature review, manuscript drafting and review; NDS, FR, DC: literature review and manuscript drafting; RR, supervision and critical manuscript revision.

Ethical consideration

Given the nature of this article, Ethical Approval was not sought.

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