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STRATEGIES TO IMPROVE FUNCTION OF MARGINAL GRAFTS AND TO RECOVER DISCHARGED GRAFTS IN LIVER AND KIDNEY TRANSPLANTATION

Presentata da: Dott.ssa Valentina Rosa Bertuzzo

Coordinatore Dottorato

Prof.ssa Annalisa Patrizi

Supervisore

Prof. Matteo Cescon

Co-supervisore

Dott. Matteo Ravaioli

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Abstract

The discrepancy between the number of transplant procedures and the patients in waiting list is linked to the scarcity of suitable donors. Dynamic perfusion of marginal grafts seems to be promising in their recovery.

Twenty discarded kidneys were used to perform a preclinical study with a new hypothermic oxygenate machine perfusion. Encouraging preclinical results allowed us to perform a clinical trial, in whom 10 extended criteria donors (ECD) livers and 10 ECD kidneys were perfused with the new machine and then transplanted, and compared with control groups (30 ECD liver transplant and 30 ECD kidney transplant preserved in cold storage).

Overall graft dysfunction was 10% in study groups, compared to 31.6% in control group ($P < 0.001$). The peak of aspartate aminotransferase within 7-days post liver transplant was 637 U/L in control group compared to 344 U/L of liver study group ($P = 0.006$). Patients receiving a kidney graft with more than 2 hours of hypothermic oxygenate perfusion treatment had lower rate of delayed graft function than control group (0% vs. 40%, $p = 0.04$).

Preclinical analyses demonstrated the absence of damage at histological examination, the improved metabolic activity and a diminished expression of genes related to ischemia/reperfusion injury, especially in case of hyperbarism application during perfusion. Further studies are needed to confirm this hypothesis. On the other hand, the clinical phase of present study documented the safety and efficacy of a new type of machine perfusion, which is low-cost and, unlike the already present in commerce, was effective and safe in both liver and kidney transplantation settings.

1. Introduction

The public announcement of the Italian National Transplant Centre reports an increase in liver and kidney transplant procedures in 2017, with 1296 liver transplants (83 more than 2016) and 1934 kidney transplants (138 more than 2016). [1]

At the same time, the donation rate increased significantly in 2017, with 2738 neurologic death certification; last year, the donor pool was composed by 1437 patients (versus 1298 in 2016).

The principal news in the Italian transplant community since 2015 is the donation after circulatory death (DCD) [2]; during the triennium 2015-2017 were registered 85 DCD. This is a difficult challenge, to whom the transplant net has answered promptly. Thanks to DCD, 68 organs has been transplanted in 63 patients since 2015.

At 31st December 2017, 8807 patients are awaiting for an organ transplant in Italy. The great majority of them is awaiting a kidney transplant (6609), while 991, 742, 354 and 262 are waiting respectively for a liver, heart, lung and pancreas transplant.

The great discrepancy between the number transplant procedures and the patients in waiting list is linked to the scarcity of suitable donors; the predicted decline in organ availability in the future, due to graft quality deterioration, is principally due to donor age and comorbidities, which are increasing simultaneously [3].

Efforts to increase the donor pool have included adding donation after cardiac death to standard donation after brain death, the "split-liver" technique, and the living donor liver and kidney transplants. Another strategy to maximize the available resources has been the inclusion of the so-called "marginal" donors in the donor pool [4,5]. The

acceptation of a graft retrieved from an expanded-criteria donor (ECD) reduces the waiting time improves the overall survival of the patient, compared to the patients who remain in list awaiting an "ideal" graft [6,7].

Nevertheless, a graft retrieved from an ECD has a major risk of a primary non-function (PNF) or a delayed-graft function (DGF); the principal cause of PNF or DGF in marginal grafts is the ischemia/reperfusion injury (IRI) which may negatively influence the functionality [8].

In synthesis, ischemia brings to ATP depletion, activation of anaerobic metabolism, acidification of the intracellular matrix, enzymatic inhibition, break of the cellular membranes with reactive oxygen species (ROS) increase in the extracellular layer and cell death due to apoptosis or cell necrosis.

During the reperfusion phase, the oxidative damage is enhanced because of the exhaustion of the anti-oxidative systems; in this moment, apoptosis prevails over cell necrosis and inflammation cells are attracted by pro-inflammatory cytokines in the damaged tissue [9].

Ischemia/reperfusion injury represents one of the principal problems for the success of organ transplant. In fact, IRI causes more than 10% of PNF and DGF. The minimization of IRI side effects should improve patient survival and, at the same time, increase the number of suitable grafts. Although is impossible to avoid IRI, because of the technique of organ retrieval, it should be diminished with rapid organ procurement, shortening of cold ischemia time and surgery procedures, but particularly, developing more effective methods of organ preservation [4,5,10].

1.2 The concept of "Expanded-Criteria Donor"

During the last decades, the concept of expanded criteria donors (ECD) has changed. Although a precise definition is still lacking, clinicians agree to define an ECD as any donor whose clinical conditions could determine a risk of graft dysfunction or diseases transmission in the transplant recipient.

In 2002, Port et al. [11] gave a precise definition of ECD for kidney grafts, which was any donor older than 60 years of age, or a donor with an age between 20 and 59 years with at least 2 of the following risk factors:

- history of arterial hypertension
- serum creatinine > 1.5 mg/dL
- cardiovascular accident as the cause of donor death.

This definition, which is valid only for kidney donors, does not consider other clinical or laboratory disorders, which could affect the quality of the grafts. Regarding abdominal organs, there is an international consensus in defining marginal a donor with the following characteristics:

- Intensive Care Unit stay with mechanical ventilation > 7 days, hemodynamic instability, need of 2 inotropes in order to maintain the mean arterial pressure > 60 mmHg;
- Body Mass Index > 30 kg/m^2;
- serum sodium peak > 165 mmol/L;
- hepatitis C or B positive serology, sepsis with bloodstream infection;
- history of previous malignant tumor, history of previous drug or alcohol abuse;

- donation after circulatory death [2,8,12].

Other patterns to define ECD are organ specific:

- donor age > 65 years, serum bilirubin > 3 mg/dL, serum transaminases > 3 times compared to normal limits, macrovesicular steatosis > 40% at pre-implant biopsy, and cold ischemia time (CIT) ≥ 12 hours, for the liver [12,13];
- in addition to Port definition [11], Karpinski score > 3 at the pre-implant biopsy, and CIT ≥ 15 hours, for the kidney [14,15].

1.2.2 Hepatic steatosis

Hepatic steatosis is frequent in deceased organ retrievals and live donors, and reported in 9%–26% of donors [12,16]. The causes of hepatic steatosis are various, including obesity, older age, alcoholism, diabetes mellitus, hyperlipidemia and nutritional changes. There are two forms of steatosis encountered in liver grafts: 1) macrovesicular steatosis (fat vacuoles occupy most of the hepatocytes cytoplasm and displace the nucleus peripherally, and is considered a more dangerous lesion); 2) microvesicular steatosis (vacuoles are smaller and have a centrilobular distribution, which is commonly found in pathological conditions associated with mitochondrial injury; it is largely reversible and does not cause harmful posttransplantation consequences). Severity of steatosis is traditionally graded as mild <30%, moderate 30%–60%, and severe >60%.

Given the steady increase in the mean age of donors and the overall increase in the prevalence of obesity, it is expected a further increase in the prevalence of steatosis in both deceased and living donors [17]. For this reason, the potential use of steatotic livers for transplant has become a major focus of investigation. However, the clinical problem

is still unresolved since steatotic livers are more subject to IRI and, when used, have poorer outcome than non-steatotic livers. Indeed, the use of steatotic liver for transplantation has been associated with increased incidence of PNF and DGF [18,19].

However, liver allografts with mild macrovesicular steatosis (<30%) can be safely used, assuming there are no other donor or recipient risk factors, because these livers show similar results to non-steatotic grafts [20]. Liver allografts with severe macrovesicular steatosis (>60%) have a significant risk of graft failure and should not be used for transplantation, unless there is an urgent situation when they are used as a bridge [16]. The use of grafts with moderate macrovesicular steatosis (30%–60%) is controversial, because these may impose a relative risk on post-transplantation outcomes.

1.2.3 Renal histology

Karpinski histological score is based on the microscopic evaluation of 4 components of the kidney parenchyma: glomerular, tubular, interstitial and vascular. The scale ascribe to every element a point, ranging from 0 to 3, where 0 is the normal histology and 3 is the maximum grade od glomerular sclerosis, tubular atrophy, interstitial fibrosis and vascular damage. The Karpinski score ranges from a minimum of 0 (indicating the absence of renal lesions) to a maximum of 12 (indicating the presence of marked changes in the renal parenchyma) [14].

In a consensus statement, an international panel of pathologists presented a method of assessing whether kidneys from a donor older than 60 years of age still contain enough viable nephrons to be made available for transplantation and whether single or dual transplantation should be used [21]. Kidneys with a Karpinski score of 3 or lower were

predicted to contain enough viable nephrons to be used as single transplants. Those with a score of 4, 5, or 6 could be used as dual transplants, on the assumption that the sum of the viable nephrons in the two kidneys approached the number in one ideal kidney. Kidneys with a score of 7 or greater were discarded, since it was assumed that they would deliver an insufficient dose of nephrons, even in a dual transplantation [14,21,22]

The importance of pre-transplant histologic evaluation has been confirmed in long-term by a prospective, multicentric, controlled, randomized clinical trial, which demonstrated that the performance of preimplantation histologic evaluation predicted better survival both in the whole study group (P=0.02) and among recipients of kidneys from older donors (P=0.01) [22]. In addition, the aforementioned study showed that graft survival of kidneys from donors ≥60 years, allocated on the basis of biopsy findings, was similar to that of single grafts from younger donors, and substantially better than that of single grafts from donors older than 60 years when those grafts were allocated on the basis of standard clinical criteria.

1.3 Pre-transplant organ preservation

The success of organ transplantation mainly depends to three factors, which are the improvement of surgery techniques, the discovery of immunosuppressive agents and the development of more effective methods of organ preservation.

Graft preservation is mandatory for every transplant program. In fact, it allows gaining time to carry the organ from the donative site to the transplant center, to find the best donor/recipient match, and to prepare accurately the recipient to surgery.

There are currently two types of preservation methods for kidneys and livers: static and dynamic. Static cold storage (SCS) is the main method for hypotermic storage, while hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) comprise the methods for dynamic preservation [23].

1.3.1 Static cold storage

Static cold storage is the clinical gold standard for preservation of most solid organs. Organs are stored in chilled dedicated preservation solutions that contain impermeants and colloids, which prevent cellular swelling and minimize molecular variations within the cells, and then put on ice. Each 10 °C drop in temperature of the organ results in a 50 percent decrease of its metabolic rate, until it reaches 10 to 12 percent of normal physiological rates at 4 °C [24].

Cell swelling, acidosis, and ROS production are primary side effects of hypothermia. Severe acidosis activates phospholipases and proteases causing lysosomal damage and eventually cell death. Therefore, the preservation solution requires pH levels to be sufficiently controlled. The first cold storage solution was EuroCollins, which uses glucose as an osmotic agent and phosphate for pH buffering [25]. The University of Wisconsin (UW) solution incorporates scavengers (glutathione, allopurinol) and adenosine as an ATP precursor. The UW solution uses Hydroxyethyl starch as a colloid to increase the oncotic pressure [26]. Another preservation solution, HTK, consists of

histidine and two amino acids, tryptophan and ketoglutarate. Tryptophan acts as membrane stabilizer, while ketoglutarate is the substrate for anaerobic metabolism during preservation [24]. Celsior is another extracellular solution and has proven to be effective in preserving abdominal grafts. It combines the osmotic control afforded by UW Solution with the buffering ability of HTK. [27].

To date, several solutions exist, with little consensus between transplant centers as to which is the best preservation solution. In spite of its successes, static cold storage does not provide long organ preservation times. The slow rates of diffusion of the preservation solutions through the organ lead to ATP depletion and necrosis within tissue.

1.3.2 Features and modalities of dynamic preservation

Lindbergh first developed the concept of dynamic organ preservation since the 1930s [28]. Thirty years later, after extensive work by pioneering groups led by Belzer [29] and Starzl [30], hypothermic dynamic preservation, using plasma or blood-based solutions perfused into the grafts, became a clinical reality. Actually, dynamic preservation was the only way to preserve organs until SCS solutions was available [25].

In recent times, with increasing use of higher risk grafts, there has been a revival of interest in dynamic preservation strategies. These strategies could offer optimized organ preservation and real-time graft viability assessment as well as a platform for delivery of conditioning agents to repair damaged organs, resulting in improved organ quality and utilization.

Perfusion requires reliable pumps, biocompatible elements of the perfusion circuit, and oxygenation and temperature control of the perfusate. During dynamic preservation,

recirculating perfusate (either acellular or blood-based) is constantly pumped through the organ vessels. Machine perfusion (MP) perfuses the organ *ex situ*, after procurement, cannulation and connection to the pump, of the graft. Continuous MP (from procurement to transplant) or pre-transplant MP (after a period of SCS) are most commonly used. The perfusate can be non-oxygenated or oxygenated. A heat exchanger regulates temperature from hypothermia to normothermia.

Hypothermic dynamic preservation aims to decrease cellular metabolism and counteract detrimental effects of ischemia. It combines low temperature (4–10°C) with the perfusion of an acellular colloid-containing preservation solution. Some evidence shows that hypothermic dynamic preservation should be controlled by pressure and not flow, using low pressures of infusion, in order to avoid pressure-related damage [31]. Pulsatile renal artery perfusion (25–30 mmHg) is best for the kidney [32,33]. The liver is perfused through the portal vein with a continuous flow that, in most circuits, is pressure-controlled (3–5 mmHg) [31,34]. It is not completely clear whether portal perfusion alone is sufficient. Maintaining the peri-biliary vascular perfusion seems to be of vital importance for the prevention of ischemic cholangiopathy. Since the hepatic artery supplies this plexus, some Authors have encouraged dual perfusion via portal vein and hepatic artery [35]. To date, clinical trials have used either single pressure-controlled portal vein perfusion [36] or non-pulsatile flow-controlled dual perfusion [34], with no significant differences in safety or efficacy.

Normothermic dynamic preservation (35–37°C) aims to restore normal cellular processes while facilitating viability assessment [37,38]. Normothermic preservation infuses an oxygenated perfusate with an oxygen carrier (usually red blood cells). Higher

pressures are used for arterial perfusion of kidney (70–85 mmHg) [39] and liver (60–105 mmHg) [40].

1.3.3 Experimental results of hypothermic machine perfusion

Kidney: It has been demonstrated the non-oxygenated hypothermic MP (HMP) of the kidney, at low perfusion pressures, can reduce DGF and may improve graft survival [41]. In fact, a randomized controlled trial comparing SCS with continuous HMP of deceased donor kidneys using the LifePort (Organ Recovery Systems, Itasca, IL) revealed a reduced risk of DGF and an increase in survival benefit, especially with ECD kidneys [42]. This portable device uses conventional roller-pump technology to generate a pressure-controlled pulsatile flow.

Recent large registry analyses (>90 000 kidneys) have shown that in standard criteria kidneys, HMP decreases the risk of DGF compared with SCS, regardless of CIT duration [43]. In the same study, the risk of DGF was reduced for ECD kidneys with CIT > 6 hours and for DCD kidneys with CIT 6–24 hours. Anyway, since CIT is a well-established predictor of DGF, a balance between minimizing CIT and any potential benefit of HMP is mandatory. Just a few hours of HMP, as long as the total CIT is not protracted, can have a positive impact on graft function compared with SCS.

Some preclinical studies have shown that cellular metabolism is slowed down but not at a standstill during HMP, resulting in oxidative stress [44]. Other recent animal studies have demonstrated that, particularly in DCD, oxygenated continuous HMP improves early kidney graft function [45]. In donation after brain death, a short period of oxygenated HMP could improve creatinine clearance compared with SCS, as effective as continuous oxygenated HMP [46].

The ideal oxygen tension, providing balance between benefit of oxygen and risk of increased production of ROS, is, so far, unknown [45,47].

Liver: Oxygenated HMP reduces IRI and protects against biliary injury in preclinical models of liver transplant; however, there is no evidence yet that HMP can extend liver CIT [48,49].

The Columbia University group was the first to report the transplantation of 20 liver grafts preserved by preimplantation HMP, compared with matched SCS controls [34]. Preimplantation HMP provided lower early allograft dysfunction (EAD) rate (5% vs. 25% of SCS livers).

Dutkowski et al transplanted 25 DCD livers after preimplantation oxygenated HMP and compared them with a matched cohort of 50 SCS DCD livers; the pumped livers showed a reduced rate of intrahepatic cholangiopathy at 1-year follow-up and improved 1-year graft survival versus SCS livers [50].

While the Columbia system used a dual flow-controlled perfusion with low pressures and continuous flow over portal vein and hepatic artery, the Zurich team applies pressure-controlled perfusion with continuous flow via portal vein.

Appropriate perfusate oxygenation seems essential to protect the liver against IRI, though the best oxygen tension is unknown.

1.3.4 Experimental results of normothermic dynamic preservation

Kidney: A short cycle of normothermic MP (NMP) prior to transplant has improved kidney graft function, replenishing ATP and reducing IRI in animal models [37]. A pilot clinical study compared 18 ECD kidneys that received 1 h of preimplantation

blood-based NMP with 47 matched SCS controls. Remarkably low DGF rates were seen with preimplantation NMP (5.6% vs. 36.2%) [39].

In contrast to HMP, kidney function can be evaluated during NMP by assessing macroscopic appearance and analysis of blood perfusate, renal blood flow and urine output [51], but proof-of-concept of transplanting kidneys that were discarded and subsequently saved by NMP has not yet been described.

Liver: NMP simulates *in vivo* conditions and requires dual perfusion through the hepatic artery and portal vein at physiological conditions.

After numerous preclinical studies showing the benefit of continuous NMP [38,52], a phase I study in the United Kingdom showed that prolonged continuous NMP is feasible and safe [53]; after a median of 9 hours of NMP, twenty livers were successfully transplanted, with 6-months survival similar to that of 40 matched controls. Bile production commenced after the first hour and was maintained throughout NMP, and median peak aspartate aminotransferase in the first 7 days was significantly lower in the NMP group (417 IU/L versus 902 IU/L, $p = 0.03$).

Another case report described a discarded liver successfully transplanted after NMP, with the choice to transplant based on normalization of lactate (< 2 mmol/L) and bile production by the graft [54]. These two values are referred as important markers, although further studies in large trials are needed to confirm their importance.

2. Purpose of the study

The main objective of the study is to increase the number of liver and kidney transplants in Italy, thanks to the application of dynamic preservation in ECD grafts. Actually, the recovery of marginal grafts represents a valid strategy to augment the donor pool and graft average.

The study is composed of 2 phases:

1) Pre-clinical phase
2) Clinical phase

In phase 1, unwanted kidney grafts, with laboratory and histological characteristics suggesting a potential recovery, will undergo dynamic preservation, in order to assess the impact of normobaric and hyperbaric oxygenation combined with hypothermia and pulsatile perfusion on the structure and metabolic activity of discarded human kidneys.

On the bases of the expertise acquired in phase 1, we will perform an interventional clinical trial to apply the hypothermic oxygenated perfusion (HOPE) in liver and kidney transplant procedure; HOPE will be performed with a machine perfusion developed in our center specifically for abdominal organ perfusion. Objective of phase 2 will be safety and efficacy of HOPE treatment in the ECD for LT and KT.

At operating speed, HOPE should increase up to 10% of organ donation, especially kidneys, decreasing waiting time for transplant, and, subsequently, reducing mortality in the waiting list.

3. Material and methods

Medica S.p.a and Centro Iperbarico S.r.l. developed an innovative organ machine perfusion, under the scientific management of our research group.

3.1 Machine perfusion: from preclinical to the clinical use

The perfusion device provides pulsatile perfusion through three peristaltic pumps in or out the hyperbaric chamber. One pump is used only in hyperbaric treatments and it breaks the gas-liquid interface facilitating the diffusion of the compressed gaseous mixture into the preservation solution. Two pumps are used to perfuse the organ through the connection between the sterile tubing (PVC and silicon) and organ's blood vessels, such as portal vein for liver and renal artery for kidney which they are cannulated with specified cannulas for organ perfusion of different vascular size (8-12 F for kidney and 18-24 F for liver).

Flow and pressure data during perfusion are detected by means of specific sensors, auto-regulated, registered on USB memory support and real-time monitored on the device's screen by the organ perfusion team. Effluent perfusate samples are collected every 15 min and analyzed to measure pO_2, pH, and lactate production.

3.1.1 Normobaric and hyperbaric oxygenation

Organ oxygenation is performed by a membrane oxygenator supplemented in the perfusion circuit (normobaric oxygenation) or a pressurizable chamber (hyperbaric

oxygenation).

Oxygen was provided to the organ preservation fluid by means of a microporous hollow fibre membrane oxygenator consisting of a gas exchange module with an integrated heat exchanger. It is a single-use and ethylene oxide sterile device, which is able to oxygenate and remove carbon dioxide from the fluid in an extracorporeal perfusion circuit until to 6 hours.

A pressurizable ("hyperbaric") chamber was designed and integrated to the perfusion circuit. During the hyperbaric treatments, each organ reservoir was placed inside the chamber, which is compressed with a mixture of carbon dioxide (5%) and medical oxygen (95%) until a higher pressure than the atmospheric one is obtained. This apparatus allows pressurization up to 200 kilopascals by a certified medical gas cylinder. The mixture is dissolved in the preservation fluid and its concentration is directly proportional to the pressure exerted by the gas on the gas-liquid interface (Henry's law). During the total organ preservation time, hypothermic or normothermic temperatures within the range of -5°C /+40°C (23/104 °F) can be guaranteed thanks to the thermal conditioning device that contains the hyperbaric chamber and can be powered from 12 to up to 220 volts.

3.2 Preclinical phase

Twenty kidneys discharged for transplant due to clinical reasons and with at least 20 h of static CIT were retrieved and enrolled in the study to test different organ

preservation strategies.

3.2.1 Study design

From May 2014 to June 2015, 20 kidneys from donors after brain death (DBD) not eligible for transplantation, and available for research aims after informed consent of the family, were randomized in the following experimental groups:

I. Static cold storage (CS);

II. Static cold hyperbaric oxygenation (Hyp);

III. Hypothermic perfusion (PE);

IV. Hypothermic perfusion in hyperbaric oxygenation (PE-Hyp);

V. Hypothermic oxygenated perfusion (PE-O_2).

Ethics Committee of University Hospital of Bologna approved the study in accordance with the institutional guidelines. According to the Starzl's technique, organ multiple harvesting was performed by our surgical team.

At the beginning, all grafts were preserved in Celsior (Waters Medical Systems of Institut Georges Lopez) at 4°C until and during surgical procedures of back-table. Renal artery was dissected and cannulated through specific cannulas such as 8 Fr, 10 Fr or 12 Fr vascular cannula (Medtronic-UK) based on vessel size. Each organ preservation treatment was accomplished at hypothermia (4°C) for 3 hours, and using 1 l of Celsior.

We set the timing of treatment at 3 hours to keep the CIT as short as possible, due to its relation to organ dysfunction and survival [15]. Additionally, in preliminary experiments longer treatment times were not associated with better features of kidneys in terms of metabolic activity and tissue damage (data not reported).

3.2.2 *Perfusion parameters in kidney preservation*

The renal flow was set in order to reach an arterial pressure of 25-30 mmHg. Oxygenation values was between 4 l and 2 l of O_2 in normobaric condition, and at 1.5 atm in hyperbaric condition to maintain pO_2 level of the preservation solution close to 750 mmHg. Pressure and oxygen parameters were established following the preliminary experience performed during machine tuning.

3.2.3 Histological analyses

The tissue alterations related to preservation damage were assessed comparing kidney biopsies prior to (T0) and after treatment (T1). Core-needle biopsies were fixed in formalin and embedded in a paraffin blocks, according to the standard procedures. Sections underwent histochemical stainings (Haematoxylin-Eosin, Trichrome stain, Periodic-acid Schiff) and microscopic examination.

In T0 and T1 tissue specimens, renal parenchyma were histologically evaluated, observing, in particular:

- Glomeruli: signs of glomerular ischemia, occurrence and diffusion of glomerular sclerosis;
- Interstitium: occurrence of fibrosis and/or inflammatory infiltrate;
- Tubules: occurrence of tubulocyte atrophy, tubulocyte vacuolization, luminal cylinders, acute tubular necrosis (ATN);
- Arteries: occurrence of myointimal thickening, detachment of endothelial cells and other signs of arterial/arteriolar damage.

3.2.4 *Immunohistochemistry (IHC)*

IHC for CD34 (mouse monoclonal antibody directed against human CD34, clone QBEnd/10, Roche Diagnostics) and CD31 (mouse monoclonal antibody directed against human CD31, clone JC70, Roche Diagnostics) was automatically carried out with BenchMark XT® immunostainer (Ventana Medical Systems, Inc, Tucson, USA) following the manufacturer's instructions.

IHC was performed on formalin-fixed paraffin embedded (FFPE) 2-µm-thick sections. Slides were dewaxed in Xylol (30 min) and rehydrated through grade washes of Ethanol: 100% (5 min), 95% (3 min) and 70% (1 min). Nuclei were counterstained with Gills haematoxylin (Sigma Chemicals). Primary antibody was obmitted in the negative controls.

3.2.5 Semi-quantitative immunohistochemistry

Immunohistochemical images were acquired using a Leitz Diaplan light microscope (Wetzlar, Germany) connected to a JVC 3CCD video camera (KY-F55B, Yokohama, Japan).

Semiquantitative analysis was performed with Image-Pro Plus® 6 software (Media Cybernetics, Silver Spring, MD, USA) using digital images taken at 10X of magnification.

Positive areas intensely stained with specific antibody, such as endothelial cell markers (CD31, CD34), were quantified selectioning at least three randomly tissue areas on average of 303622.56 μm^2. To accomplish a comparable selection, only images that exclusively contained a single glomerulus were quantified (as seen in Figure 1, right column).

3.2.6 Transmission Electron Microscopy (TEM)

TEM analysis were performed using small tissue fragments fixed in Karnowsky fixative (2% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer), and processed according to the local protocol.

Each sample was rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer at room temperature for 1 hour, dehydrated with graded ethanol (from 30 to 100%) and embedded in Araldite resin. Uranyl acetate and lead citrate have been employed to counterstain the ultrathin sections. Ultrastructural examination of tissue specimens was carried out on Philips CM10 (FEI Company, Milan, Italy) transmission electron microscope equipped with a Gatan camera; digital images were captured using the FEI proprietary software Olympus SIS Megaview SSD digital camera.

3.2.7 Metabolic analyses

Perfusate samples were collected from the organ reservoir at T0 and T1 and assessed to detect pH, lactate concentration, oxygen (pO_2) and carbon dioxide (pCO_2) partial pressure by means of a haemogasanalyzer (Gem Premier 3500, Instrumentation Laboratory-Werfen s.p.a., Barcelona, Spain).

3.2.8 *Tissue ATP content evaluation*

At T0 and T1, tissue samples were collected and immediately frozen in liquid nitrogen. Following protein extraction, ATP content was detected using an ATP determination kit (Cat. N°A22066, Thermo Fisher, Waltham, Massachusetts, USA).

Proteins extraction was accomplished using a buffer containing 150mM NaCl, 20mM Na_2HPO_4/NaH_2PO_4, 10% glycerol, 1%Triton X-100, PMSF 100mM, DTT 100mM and a mix of protease inhibitors (Roche; Basel, Swiss). According to the Lowry

method, total protein concentration of each sample was determined and then diluted to a final concentration of 1 µg/µl. The tissue ATP content was determined from five micrograms of total protein on a Glomax 20/20 single tube luminometer *(*Promega, Fitchburg, Wisconsin, USA*)* following the manufacturer's instruction. Finally, each absolute ATP concentration was calculated by interpolating the luminescence value on a standard curve, and then the ATP value was expressed as ratio of T1/T0 (Figure 3).

3.2.9 Gene expression analysis

Total RNA was extracted from each tissue sample that it was collected in liquid nitrogen. The Trizol RNA isolation protocol and reverse transcribed by using a SuperScript Vilo Master Mix (Invitrogen; Waltham, Massachusetts, USA) were applied before cDNA amplification. A semi-quantitative Real-time qPCR on an iCycler (Biorad; Hercules, California, U.S.A.) with SYBR GreenER qPCR SuperMix (Invitrogen, Waltham, Massachusetts USA) was accomplished using the following primers: Hypoxia Inducible Factor 1 alpha (HIF-1α) (FW AACATAAAGTCTGCAACATGGAAG; RV TTTGATGGGTGAGGAATGGG), Endothelial Nitric Oxide Synthase (eNOS) (FW GTGGCTGGTACATGAGCACT; RV GTCTTTCCACAGGGACGAGG), Beta Actin (FW CCTGGACTTCGAGCAAGAGATG; RV GGAAGGAAGGCTGGAAGAGTG**)** and Beta-2Microglobulin (FW CTTTCTGGCCTGGAGGCTATC; RV CTTTCTGGCCTGGAGGCTATC).

Data were analyzed according to the $2^{-\Delta\Delta ct}$ method using the expression of each gene at T0 as the reference value. Therefore, gene expression level was reported as fold induction of T1/T0 (Figure 4a - 4b).

3.3 Clinical phase

3.3.1 *Study design*

This national, single-center, prospective and interventional clinical trial was performed at the General Surgery and Transplant Unit of University Hospital of Bologna, S. Orsola-Malpighi Polyclinic, according to the Helsinki Declaration and following ethical approval from the local committee (Number of clinical trial protocol registration, ClinicalTrials.gov ID: NCT03031067).

Liver (N = 10) and kidney recipients (N = 10) on Bologna transplant waiting list were selected for enrollment study in the period October 2016 - December 2017. All recruited recipients were transplanted with ECD graft, liver (HOPE-L group) or kidney (HOPE-K group) that was restored in the pre-implantation phase by HOPE.

HOPE-L and HOPE-K groups were compared to the matched control groups of liver and kidney recipients, which were transplanted from January 2004 to September 2017 at the same transplant center with organs preserved by SCS, SCS-L and SCS-K groups respectively.

Data collection about demographic, clinical, organ preservation, histological and transplant outcome was accomplished prospectively for study group and retrospectively for control group.

3.3.2 Matching study and control cases

Each study case of HOPE-L and HOPE-K groups was matched 1:3 to the control cases of SCS-L and SCS-K groups. The case selection of SCS-L and SCS-K recipients was carried out using specified donor and recipient clinical characteristics.

HOPE-L and SCS-L cases were matched for:

- donor and recipient age;
- CIT;
- MELD (model of end-stage liver disease) score;
- previous abdominal surgery;
- portal thrombosis;
- hepatic steatosis 0-10% or 10-30%.

HOPE-K and SCS-K cases were matched for:

- donor and recipient age;
- CIT;
- total Karpinski's score;
- dialyses type (peritoneal or hemodialysis);
- dialyses time.

3.3.3 *Recipient and donor selection*

ECD donors aged over 18 years and DBD were considered as suitable for the study inclusion and they were enrolled after the signature of the informed consent by the family. According to the UNOS criteria, donors were defined ECD as follows:

- liver: donor is marginal if he/she had hemodynamic deterioration, age > 65 years, BMI > 30 kg/m^2, bilirubin > 3 mg/dl, AST o ALT above three times the upper reference threshold, sodium > 165 mmol/l, days on intensive re unit (ICU) > 7, steatosis > 40%, cold ischemia time (CIT) >14 h) [12,13];

- kidney: donor is marginal if he/she aged ≥ 60 years or aged 50-59 years with two or more other risk factors such as cerebrovascular accident, hypertension and serum

creatinine > 1.5 mg/dL [11].

Following the signature of the informed consensus, recipients aged over 18 years on liver/kidney waiting list to University Hospital of Bologna and eligible by donor matching were recruited in the trial.

Donor or recipient with vascular anomaly, urgency transplant, re-transplantation and combined organ transplant, were excluded from the study.

3.3.4 *Organ retrieval and transfert*

The livers and kidneys harvest were performed according to the standard procedures and our ECD policy [4,55,56].

Following the organ retrieval, a cooling sterile bag with new preservation solution was used to store the graft during the transfer to the transplant hospital.

3.3.5 *Liver graft histopathological analysis*

The homogeneity of the study population between HOPE-L and SCS-L groups, as well as between HOPE-K and SCS-K groups, was investigated comparing graft histopathology, which was revised in all cases when the allocation biopsy had been performed.

Liver biopsies were sent to out Transplant Pathology Unit without any fixation media, for the frozen-section analysis for graft suitability. After the frozen sections, tissue was fixed in formalin, embedded in paraffin and routinely processed. From paraffin blocks, 2-µm-thick sections were cut for permanent Haematoxylin-Eosin and Reticulin stains.

In liver grafts, according to the guidelines adopted in our Institution [57], 9 variables were separately evaluated:

- Percentage of macrovesicular steatosis;
- Percentage of microvesicular steatosis;
- Portal fibrosis (stage) according to METAVIR [58];
- Amount of portal inflammatory infiltrate according to Ishak [59];
- Amount of lobular necrosis/inflammatory infiltrate according to Ishak;
- Arteriolar myointimal thickening, scored as absent, mild, moderate and severe, with eventual lumen obliteration;
- Biliocyte regressive changes, scored as absent, focal or diffuse;
- Ductular reaction/neo-ductulogenesis, scored as absent or present;
- Cholestasis, scored as absent or present.

3.3.6 Kidney graft histopathological analysis

Kidney biopsies were sent to out Transplant Pathology Unit in Serra solution for the histological analysis for graft suitability. Tissue was rapidly processed in microwave oven. From paraffin blocks, 2-µm-thick sections were cut for permanent Haematoxylin-Eosin, Masson's Trichrome and Periodic Acid Schiff stains. Thirteen variables were separately evaluated:

- Glomerulosclerosis, according to Karpinski [14];
- Tubular atrophy, according to Karpiski [14];
- Interstitial fibrosis, according to Karpiski [14];
- Vascular damage, according to Karpiski [14];
- Total Karpinski's score [14];
- Glomerular ischemic changes, scored as absent, focal or diffuse;
- Mesangial matrix thickening, scored as absent, mild or moderate;
- Presence of thrombotic microangiopathy [60];

- Acute tubular necrosis, scored as absent, focal or diffuse [61];
- Isometric vacuolization of tubulocytes, scored as absent, focal or diffuse;
- Presence of intraluminal calcifications of tubuli;
- Prevalence of sclerotic or hyaline changes in arterial/arteriolar walls;
- Presence of interstitial inflammatory infiltrate.

3.3.7 Hypothermic oxygenated perfusion

Ex-vivo hypothermic oxygenated perfusion was performed through our innovative machine perfusion, already tested in the preclinical study, after the notification to the Health National System for its clinical use in organ perfusion [62].

HOPE was performed at 4°C, with an oxygen partial pressure (pO_2) of 600-750 mmHg, with Belzer University of Wisconsin machine perfusion solution, a portal vein pressure of 5 mmHg and renal artery pressure of 25 mmHg.

During all surgical back-table time, HOPE started with flushing mode at controlled pressure, as mentioned above, flow of 30 ml/min and new perfusion solution continuously. At the end of the surgical graft preparation (average time of 30-40 minutes), HOPE continued normally recirculating the same perfusion fluid, at controlled pressure and without limits on flow parameters. Effluent perfusate samples are collected at T0 and T1 to accomplish microbiological analyses and every 15 minutes to measure pH, pCO_2, pO_2 and lactate. The microbial cultures on perfusion fluid before (T0) and after HOPE (T1) were performed to test any contamination related to the HOPE treatment, and haemogasanalyses to assess the oxygen supply and the lactate production as a metabolic marker.

3.3.8 Transplant procedure

According to the standard techniques, surgical team accomplished kidney and liver transplants.

Kidneys were implanted into iliac fossa anastomosing the artery to the external/common/internal iliac arteries, the vein to the external/common iliac veins and ureter-bladder over a single stent.

Livers were transplanted in orthotopic position, with the piggyback technique [63].

3.3.9 Short and long-term post-transplant follow-up

Post-transplant medical care including antimicrobial/antifungal and antithrombotic prophylaxis, has been managed following the standard protocols [4,5,64,65].

According to the international recommendations, KT immunosuppressive therapy was induction therapy and tacrolimus plus steroids, with or without mycophenolate, as either mycophenolate mofetil or mycophenolate sodium steroid dose decreasing.

LT immunosuppression protocol was based on tacrolimus in combination with low-dose steroids. Induction therapies in combination with tacrolimus were administered in selected patients with autoimmune diseases, pre-LT renal insufficiency, or positive cross-match.

Post- transplant follow-up was recorded at 1-, 3-, 6- and -12 months.

3.3.10 Transplant outcome

Graft survival, PNF and EAD or DGF incidences were determined to evaluate the HOPE effects in ECD DBD LT and KT, compared to SCS.

Organ failure, leading to re-transplant for LT, or constant dialyses recovery/transplantectomy for KT, was identified as PNF.

Bilirubin >10 mg/dL on post-operative day 7 or/and INR >1.6 on post-operative day 7 or/and aminotransferase level (alanine aminotransferase, ALT, or aspartate aminotransferase, AST) > 2000 IU/mL within the first 7^{th} post-operative day, were considered to define EAD event in LT [66].

Dialysis need within the first week after KT was defined as DGF [67].

Furthermore, graft functional parameters at 1-, 3-, 6- and -12 months post-transplant, biliary/ureteral fistula, graft rejection and hospital readmission until one year post-transplant, were assessed and compared between HOPE and SCS groups.

3.4 Statistical analysis

Continuous data are presented as the ratio between values measured at T0 and T1 or absolute value, mean ± SD or median with range); categorical data are expressed as percentage values. Kolmogorov-Smirnov test was used to test the normality of the distribution while the homogeneity of variance was evaluated through the Levene test. Differences between groups were evaluated by means of parametric (ANOVA, T test) or non-parametric (Mann-Whitney U test) analyses. Data correlation among different study parameters was performed following Pearson's method. Kaplan-Meier was applied to perform the survival analysis.

SPSS version 20.0 and GraphPad Prism 5.0 software were used for statistical analysis. A p value < 0.05 was considered statistically significant.

4. Results

4.1 Preclinical study results

4.1.1 Features of the discarded kidneys

The 20 kidneys were discarded for various reasons: high Remuzzi score [22], donor age, or too long CIT. The mean donor age was 70 ± 4 year while the minimum CIT before the start of the planned treatment was 20 h.

Data are comparable among all experimental groups (Table 1).

4.1.2 Histological analysis and immunohistochemistry

Each experimental group included organs (minimum one and maximum three) at T0 with pathological evidences of glomerular sclerosis, tubular injury, mild interstitial fibrosis, interstitial chronic fibrosis or arterial myointimal thickening. After T1, endothelial damage appeared in one or two cases for all groups without any correlation to perfusion or oxygen.

An increased expression of CD31 and CD34, indicating a well-preserved tissue, was observed in the CS, Hyp and PE-Hyp groups; instead, expression was not significant for PE and PE-O2 (Figure 1, left column). TEM showed endothelial injury only for the PE-group (Figure 2C).

Here we report in detail the histological analysis and immunohistochemistry of the

five experimental groups:

I. Static cold storage (CS)

The four grafts of the control group had mainly mild interstitial fibrosis, tubular atrophy and glomerular sclerosis at T0.

After CS (T1), 2/4 grafts showed a histologically visible tubular injury, characterized by simplification and focal vacuolization of tubulocytes, and appearance of ATN was observed in two cases. One graft showed endothelial damage. At T1, immunohistochemistry showed an increased expression of the investigated proteins (Figure 1, left column); in particular, CD34 increased significantly. These results, indicating well preserved tissue, were confirmed by ultrastructural examination; indeed, in T1 samples, endothelial cells of glomeruli and *interstitium* showed excellent morphology, including nuclei with dispersed chromatin and small nucleoli as well as a normal complement of cytoplasm organelles (Figure 2A).

II. Static cold hyperbaric oxygenation (Hyp)

Among the four grafts of this group, one had some degree of tubular injury with ATN at T0.

At T1, three grafts showed the same morphological picture as to T0 and endothelial damage appeared in one graft. The expression of CD31 and CD34 increased (Figure 1, left column); a significant CD34 increase was seen. At ultrastructural examination, endothelial cells were normal without any feature of subtle cell damage (Figure 2B).

III. Hypothermic perfusion (PE)

Among the four grafts of this group, 2 had some degree of tubular injury with ATN at T0, and diffuse glomerular sclerosis was seen in one. After PE, 2/4 grafts showed the same morphology as T0, while 2 grafts had some degree of endothelial injury.

Unlike the previous group, a decreased and non-significant expression of CD31 and CD34 was seen at immunohistochemistry (Figure 1, left column). TEM showed endothelial cell injury, markedly electron dense shrunken cells with cytoplasm vacuolization (Figure 2C).

IV. Hypothermic perfusion in hyperbaric oxygenation (PE-Hyp)

In this group, only one case was characterized by diffuse glomerular sclerosis, tubular atrophy with interstitial chronic flogosis and arterial myointimal thickening. At T1, two grafts showed the same morphology as at T0, while the other two showed a global worsening of histologic appearance, with glomerular simplification, tubular atrophy and endothelial loss.

The CD31 and CD34 expression increased in this group too, and CD31 was significantly expressed after the treatment (Figure 1, left column). Accordingly, TEM revealed unremarkable endothelial cells (Figure 2D).

V. Hypothermic oxygenated perfusion (PE-O2)

Among the four grafts of this group, three showed some degree of tubular injury with ATN at T0.

After PE-O2, 3/4 grafts showed the same morphology as at T0, while one graft had some degree of endothelial injury. The CD31 and CD34 expression did not vary after the treatment; in particular, CD31 showed a slightly increased expression whereas CD34 decreased a little (Figure 1, left column). TEM of endothelial cells did not show any difference after the treatment (Figure 2E).

4.1.3 Metabolic evaluation of the preservation solution

As expected, pO_2 was significantly higher in the Hyp, PE-Hyp and PE-O2 groups compared to the CS and PE groups. At T1, the lactate concentration was significantly higher in all perfusion groups except for PE-Hyp. The pCO_2 was significantly higher in the Hyp and PE-Hyp groups with respect to all the other groups. Of note, pCO_2 levels were not evaluated in the PE-O2 group since the oxygenator was equipped with a CO_2 scavenger. Data, expressed as mean and SD, are reported in Table 1.

4.1.4 *Tissue ATP content*

The amount of ATP content for each preservation group is shown in Figure 3, and presented as the ratio of T1 over T0 samples.

In the CS, Hyp and PE groups, a net depletion of ATP content following the preservation procedure was observed. On the contrary, PE-Hyp as well as PE-O2 were associated with a net increase of ATP content with respect to baseline levels. As a result, at the end of the preservation the amount of ATP was significantly higher in the PE-Hyp group with respect to the CS, Hyp and PE groups, while ATP level in PE-O2 was significantly increased only with respect to the PE and Hyp groups.

Additionally, grouping all samples with the exception of PE-O2 for the CO2 of the oxygenator device scavenger showed a significant correlation between ATP content and pCO2 (Pearson correlation 0.759, P=0.001).

4.1.5 Gene Expression of eNOS and HIF1α

As shown in Figure 4A, the mRNA level of eNOS was reduced in the PE-Hyp group, while in the other groups, including PE-O_2, the mRNA level was not decreased. As a result, PE-Hyp was associated with a significant down regulation of eNOS gene expression with respect to CS, PE, Hyp and PE-O_2 groups.

The gene expression of HIF-1α was also evaluated. All preservation modalities were associated to a slight reduction in the expression of HIF1α with respect to CS, reaching statistical significance only in the PE-Hyp group (Figure 4B).

Expression of genes involved in the inflammatory response (interleukin-6) and cellular apoptosis (caspase-3) were also evaluated; however, no significant differences between groups were seen when the gene expression levels in T1 samples were compared to that of T0 samples (data not shown).

4.2 Clinical trial results

4.2.1 Donor and recipient characteristics

Between 6 October 2016 and 19 December 2017, N = 20 recipients (N = 10 LT recipients and N = 10 KT recipients) were consecutively recruited and transplanted with

ECD DBD donor organ preserved from surgical back-table to implantation by HOPE (Table 2).

Recipients of liver transplants had hepatocellular cancer on cirrhosis with one or more etiology: alcoholic liver disease (N = 2), hepatitis C virus infection (N = 5); hepatitis B and D virus infection (N = 2); hepatitis B virus infection (N = 1); primary biliary cirrhosis (N = 1); one patient was affected only by alcoholic cirrhosis. Recipients of kidney transplants had end-stage renal disease with the following etiology: interstitial nephritis (N = 1), renal polycystosis (N = 2), hypertensive renal vascular disease (N = 1), diabetic nephropathy (N = 1), hypertensive nephron-sclerosis (N = 2), membranous glomerulonephritis (N = 1), nephron-angiosclerosis (N = 1), chronic glomerulonephritis (N = 1).

The study population was properly matched with the control group (Table 1) and none differences were found between the two groups. L-HOPE and L-SCS groups were matched similarly for MELD score, previous abdominal surgery, portal thrombosis and macrovesicular hepatic steatosis of the graft. In the HOPE-K group, one case had double KT which was matched with 3 double renal transplant cases of the control group. Lastly, type and time dialyses were also equally dispensed between study and control group.

Median donor age was very high: 77.5 (60-84) years for HOPE-L group and 71.5 (60-78) years years for HOPE-K group, compared to 75.5 (53-85) years for SCS-L group and 69.5 (59-79) years for SCS-K group (p=n.s.).

In the L-HOPE group, the median CIT was 7.1 (6.1-9.6) hours: a value time comparable to 7 (5.4-10) of L-SCS group and very low because the perfusion treatment started at the time of the organ back-table and the livers were immediately transplanted. The median CIT for the kidney group was 14 hours because in our centre we did not perform KT during the night. However, this data was still comparable among the HOPE-

K and SCS-K groups, 14.5 (10.8-22) hours versus 14 (8-21) hours respectively.

Induction immunosuppressive therapy was a matching criteria which it was distributed not uniformly between K-HOPE and K-SCS groups; indeed, anti-thymocite globulines and basiliximab was administered to 50% of HOPE-K cases but K-SCS cases received anti-thymocite globulines for 16.7% and and basiliximab for 83.3%.

4.2.2 *Histopathological features*

Liver histology was available in 25 cases, 9 HOPE-L and 16 SCS-L. Table 3 illustrates the distribution and percentages of the 9 variables evaluated in HOPE-L and SCS-L. No statistically significant differences were found between the two study groups.

Kidney histology was available in 38 cases, 11 HOPE-K and 27 SCS-K. Table 4 illustrates the distribution and percentages of the 13 variables evaluated in HOPE-K and SCS-K. Median of Karpinski score was 3 (0-6) in SCS-K group and 4 (0-7) in HOPE-K group. No statistically significant differences were found between the two study groups.

4.2.3 Perfusion parameters and biochemical characteristics of the HOPE perfusates

The perfusion parameters of N = 11 kidneys, N = 9 for single KT and N = 2 for dual KT, and N = 10 livers undergoing HOPE before implantation are reported in Table 5. None accident event of organ perfusion was registered for study cases enrolled.

The median hepatic portal flow was 107.5 (65-116) ml/min and median perfusate lactate levels was 1.8 (1-3) mmol/L; the median renal flow was 52.5 (24-85) ml/min and median perfusate lactate levels was 1.3 (0.7-2.5) mmol/L.

Oxygen level was in line to the reference limits during HOPE, as demonstrated by the pO_2 values at T1 (Table 5).

The median perfusion time was 2.2 (1-3.5) hours for HOPE-L group and 3.3 (1-6)

hours for HOPE-K group.

Bile or urine production were not assessed during liver or kidney perfusion treatment, respectively.

Microbiological analysis of the perfusate specimens did not revealed any bacterial or fungal growth related to the perfusion strategy under study.

4.2.4 *Graft function and post-transplant complications*

Data to assess the graft function were summarized in the Table 6.

Overall graft dysfunction was 10% in the study groups, compared to 31.6% in the control group ($P < 0.001$). PNF was null for both KT-groups, and zero for HOPE-L versus 6.6% for SCS-L ($P = 0.086$).

L-HOPE group had 0% of EAD versus 20% of L-SCS group. As result of this data, the peak of aspartate aminotransferase (AST) within 7-days post-LT was 637 (124-2100) U/L in L-SCS group compared to 344 (166-1032) U/L of L-HOPE group ($P = 0.006$). INR were significantly lower in L-HOPE recipients compared to the L-SCS group ($P= 0.04$).

Concerning the kidney group, the study population showed a trend to better outcome, with only 20% of DGF compared to 40% in the control group. The mean value of creatinine in 5^{th} post-operative day was 3.5 ± 2 mg/dL for K-HOPE patients and 4.1 ± 2.6 mg/dL for K-SCS. DGF events occurred in the 2 cases in which the perfusion time was less than 2 hours; the remaining 8 cases in HOPE-K group had no DGF. Comparing K-HOPE patients receiving a graft with more than 2 hours of perfusion treatment to the control group, the rate of DGF was statistically different (0% vs. 40%, $p = 0.04$).

Post-operative complications, according to the Dindo-Clavien classification [69], were comparable among the study groups, with any statistical significance (Table 7).

4.2.5 Biliary/ureteral strictures and organ rejection

Biliary and ureteral strictures were recorded until 1 year post-transplant; no differences were found for HOPE recipients versus SCS group [L-HOPE group = 1/10 (10%) versus L-SCS group = 3/30 (10%); K-HOPE group = 1/10 (10%) versus K-SCS group = 2 /30 (6.6%)].

Likewise, the incidence of acute or chronic rejection was similar between study and control groups [L-HOPE group = 1/10 (10%) versus L-SCS group = 4/30 (13.3%); K-HOPE group = 1/10 (10%) versus K-SCS group = 2/30 (6.6%)].

4.2.6 Hospital stay, graft and patient survival

There were no differences in length of hospitalization for LT patients (11 days for L-HOPE versus 12 days for L-SCS) and for kidney transplant (17 days for K-HOPE versus 24 days for K-SCS).

All grafts and patients of HOPE groups were alive 1-year post-transplant. In L-SCS group, graft survival was 90% due to two PNF events and one case of multiorgan failure following sepsis and patient survival was 90% due to three death events (one for infectious complications and two for recurrence of livers disease). In the K-SCS group, graft survival was 93.3% due to two graft failure cases (one for renal vein thrombosis in a single KT and one of multiorgan failure following bacterial sepsis in a dual KT), and patient survival was 96.6% due to one death for infectious complication (Table 6).

5. Discussion

The preclinical phase of this study indicated that three hours of hypothermic perfusion with hyperbaric or normobaric oxygenation was able to restore ATP levels, a signal of organ functional activity [70]. These improvements were possible even with extended criteria grafts declared not suitable for transplantation, and after more than 20 hours of CIT.

Metabolic evaluations showed that the high pO_2 level detected at T1 in the Hyp, PE-O_2 and PE-Hyp groups was related to a higher level of pCO_2 for Hyp and, mostly, for the PE-Hyp group, showing an increased metabolic activity of the graft. Therefore, the different pCO_2 level between the Hyp and PE-Hyp groups suggested that hyperbaric oxygenation without perfusion may lead renal cells to not efficiently exploit the O_2 dissolved in the storage solution to maintain their basal metabolism. A lower pCO_2 level was detected in the PE-O_2 group, due to the CO_2 scavenger of the oxygenator device. In the PE group, pCO_2 did not change at T1 with respect to T0; only perfusion was less effective in maintaining the renal basal metabolism.

PE and PE-O_2 had significantly higher levels of lactic acid in the preservation solution at T1, which is in contrast with the corresponding value in PE-Hyp group. During perfusion, the hyperbaric oxygenation probably stimulated aerobic metabolism, yielding higher ATP and lower lactate concentrations, as previously reported by other Authors in different experimental settings [71-72]. The reduced lactate production may contribute to the beneficial effect of the hyperbaric oxygenation in IRI.

Furthermore, hyperbaric perfusion showed a significantly decrease in gene

expression of HIF-1α and eNOS. These results again suggested a reduction in preservation injury and a better delivery of oxygen to the tissue. In fact, HIF1α expression normally occurs during SCS as an adaptive tissue response to sub-optimal oxygen tension; its reduced expression in the PE-Hyp group indicated an optimal oxygen delivery to the graft [73]. Indeed, it was reported that eNOS gene expression is upregulated during hypoxia, thus favoring vasodilation, in order to ensure an adequate blood supply [74].The reduced expression of eNOS in the PE-Hyp group supported this hypothesis.

Our preclinical study explored only some aspects of the beneficial effects of dynamic perfusion, but the absence of damage at histological examination, the improved metabolic activity and a diminished expression of genes related to IRI, appear as good basis for future application of PE-Hyp in clinical settings.

The expertise and the encouraging results reached in the preclinical phase encouraged us to establish the clinical trial. The greater international experience with hypothermic perfusion for ECD grafts [31-36,41-50], associated with the difficulty in developing a portable hyperbaric chamber for clinical use, moved us to perform HOPE in marginal liver and kidneys.

Although metabolically dormant allografts have a low oxygen demand, the impaired mitochondrial capacity inherent to marginal grafts may require a higher oxygen supply to induce an active forward metabolism of accumulated succinate, which ultimately leads to a reconditioning effect and ATP resynthesis [75-76]; hypothermic oxygenated machine perfusion give the oxygen supplementation that ECD grafts needs. The protective effects of HOPE have been confirmed in an animal model of LT for fatty donor organs, in which IRI, oxidative stress, Kupffer and endothelial cell activation were reduced in steatotic liver grafts treated with HOPE, compared to SCS [77].

The clinical experience in hypothermic oxygenated perfusion, at the moment, is limited to liver grafts [36,50,75-76], while this technique is still used in preclinical model of kidney transplant [45-47]. We recently reported the efficacy of HOPE in restoring s histologically discarded pair of kidney, which were successfully transplanted [78].

The present work suggests that the newly developed hypothermic oxygenated pump used in this study was effective and safe in both liver and kidney transplantation settings. The principal innovation that we propose, in respect with other previous study about HOPE and ECD [50,75-76] was a perfusion machine effective for both liver and kidney. The system was easy to use and it allowed us to start HOPE during back-table procedures, decreasing cold storage period.

Many previous studies [34,41,43-45] suggested to start the hypothermic oxygenation of the graft immediately after retrieval, in order to reduce the accumulation of waste products, such as succinates, which leads to enhanced restoration of the mitochondrial function [71-72,77]. Our protocol started with a graft flushing for 30-40 minutes during back-table; the system was settled to avoid fat embolism and to remove completely the first part of the preservation fluid flushed. After this step, oxygenation and recirculation of the preservation fluid started. We believe this strategy may substitute the necessity to start immediately the graft perfusion at the end of the retrieval, if the cold ischemia time was maintained low, as in our study.

We did not register adverse events related to our system; however, the outcome was excellent for the liver grafts without any dysfunction despite the very old age of the donors. Regarding the kidney transplantation group, we did not lose any graft and only two cases had a graft dysfunction, but evaluating the perfusion time, these two cases were perfused for less than two hours. Effectiviously, the rate of DGF of patients receiving a

graft with more than 2 hours of perfusion treatment was statistically different in respect with the control group (P= 0.04). Since our study is the first in which HOPE is applied to ECD kidney grafts, we used the same strategy as for the liver grafts, where we perfused at least one hour and then we transplanted the liver as soon as possible. Our data suggested that should be necessary at least two hours of HOPE for the kidneys, but future randomized trials are needed to confirm the present hypothesis.

Many previous studies, even from our centers [62,75-76] showed that the hypothermic oxygenation was able to restore the ATP in the liver and kidney grafts, reducing theoretically the graft dysfunction after transplantation. Our clinical trial showed a better liver function in the perfused liver, confirmed by a lower peak of transaminases a lowed number of cases of EAD. In effect, we reached the same end-point reported by Nasralla et al. [79], who used normothermic perfusion starting at the time of retrieval. The transaminases level were reduced at the same way, even if our donors were much older.

The principal limit of our results was the lack of any metabolite measurements in the perfusion fluid from the grafts; so we could not find a biochemical explanation to our theory. On the other hand, our study objective was to evaluate the clinical safety and efficacy of a new hypothermic oxygenated perfusion system.

In conclusion, our study revealed that:

- hypothermic, hyperbaric or normobaric oxygenation, dynamic perfusion is should improve organ metabolic preservation compared to other treatments. In this context, the absence of damage at histological examination, the improved metabolic activity and a

diminished expression of genes related to IRI, appear as good basis, in particular, for future application of hyperbarism in clinical settings of graft preservation;

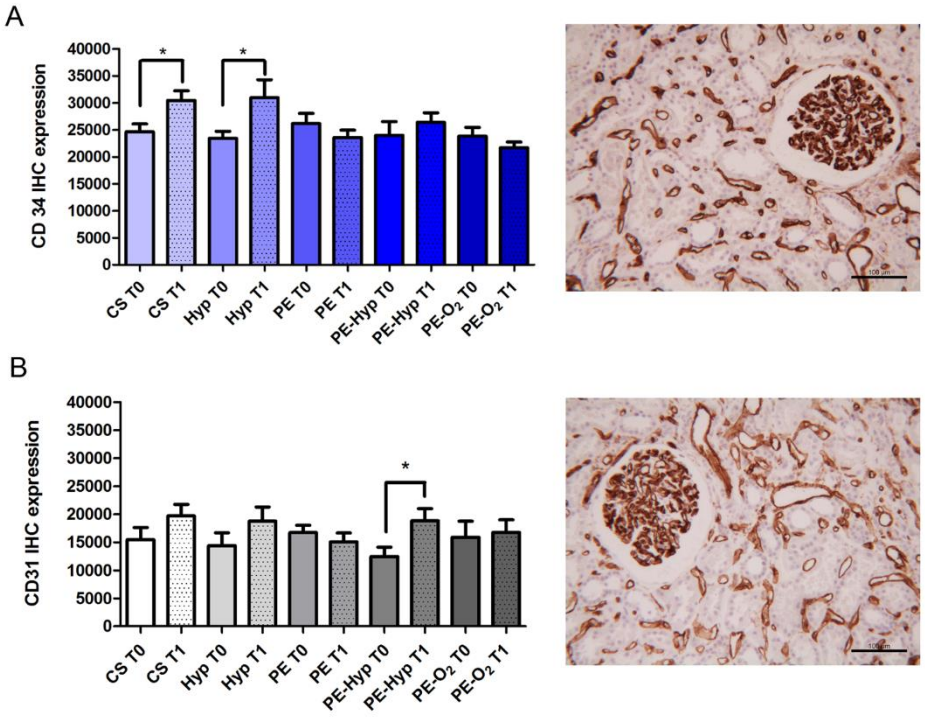
- the new hypothermic oxygenated perfusion system that we developed is safe and effective for clinical application;

- HOPE should be performed for at least two hours for kidney grafts from ECD;

- HOPE determines a better liver function in ECD liver grafts; early allograft dysfunction is reduced with hypotermic oxygeneated perfusion as well as with normothermic perfusion.

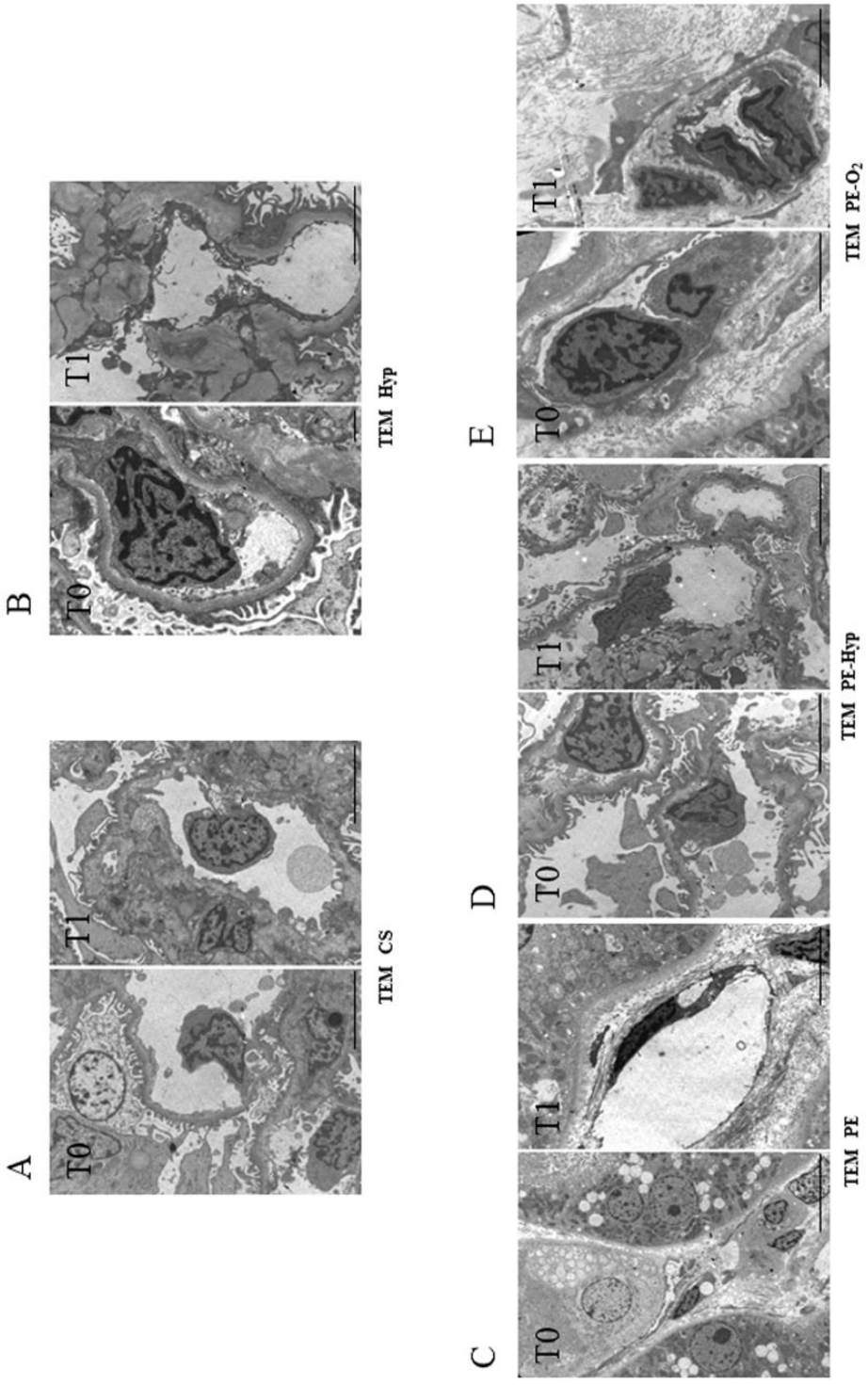
6. Figures and tables

Figure 1. Representative immunohistochemical images (right column) and semiquantitative analysis (left column) of kidney tissue expressing CD34 (A) and CD31 (B) endothelial cell markers.



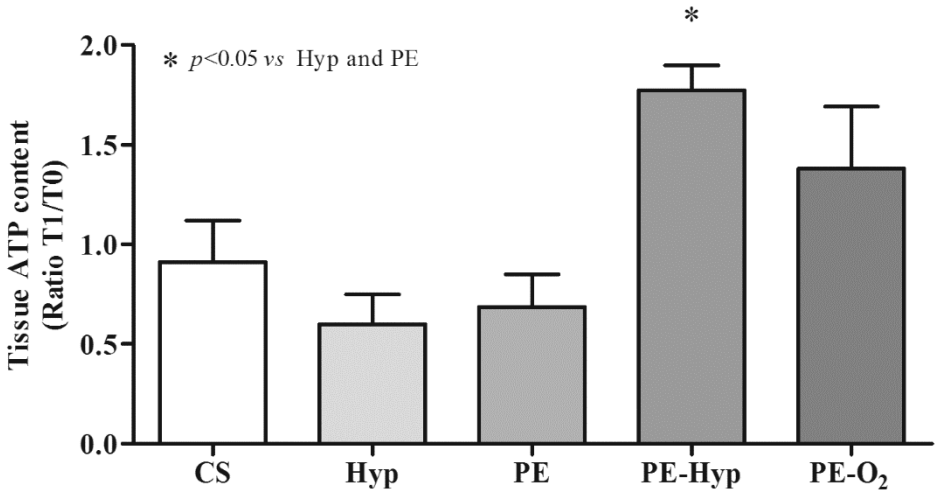
A-B: scale bars = 100 µm. A: * p-value = 0.0187 for CS group, * p-value = 0.0466 for Hyp. B: * p-value = 0.0109 for Hyp-PE group; unpaired Student's t-test.

Figure 2. Representative ultrastructural images of endothelial cells seen in glomerular loop and *interstitium*.



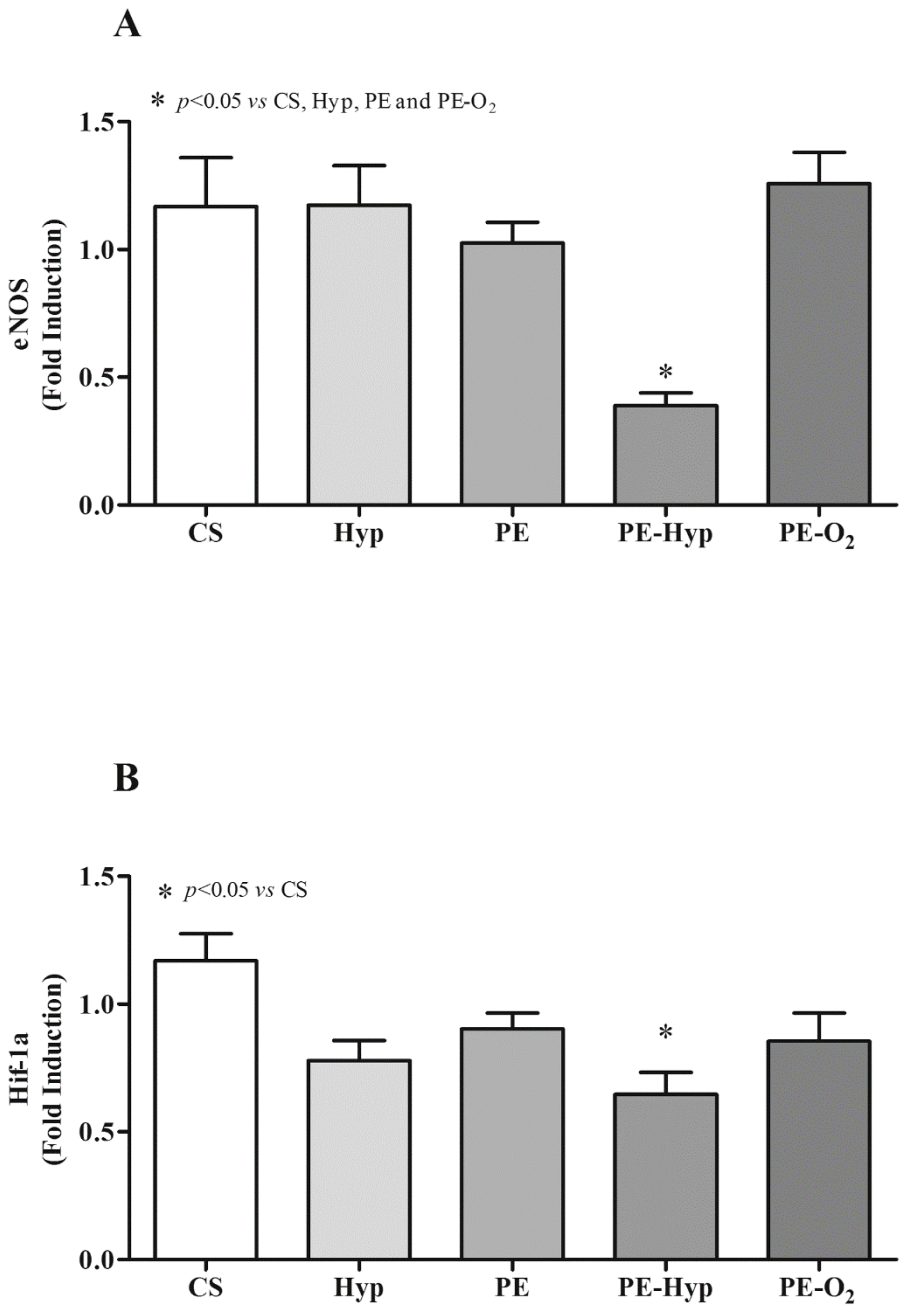
A: Scale bars = 5 µm; B: T0 scale bar = 2 µm, T1 scale bar = 5 µm; C: T0 scale bar = 5 µm, T1 scale bar = 5 µm; D-E: scale bars = 5 µm.

Figure 3. Tissue ATP level.



Results are expressed as the ratio between ATP content at T1 and T0. The results of post-hoc analysis are indicated by asterisk.

Figure 4. Gene expression of endothelial nitric oxide synthase (eNOS) (A) and hypoxia inducible factor 1α (HIF-1α) (B).



Data presented are fold induction of sample at T1 over T0. The results of post-hoc analysis are indicated by asterisk.

Figure 5. Peak of aspartate aminotransferase within 7 days after liver transplant in study group (HOPE) compared to the control group (SCS).

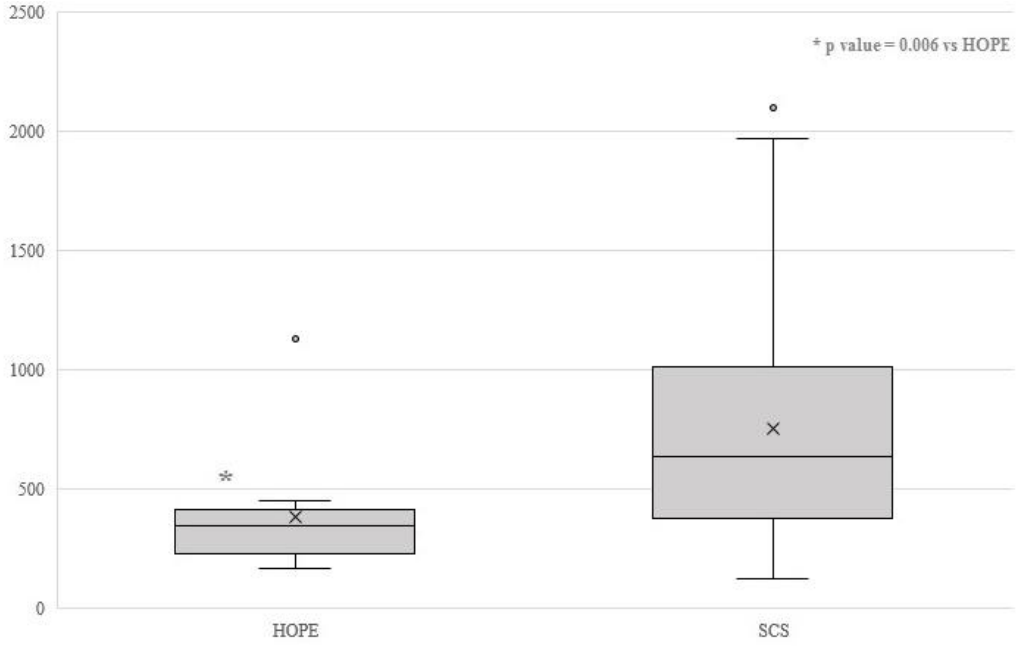


Table 1. Features of the discharged kidney enrolled in the five experimental groups

	CS (4)	Hyp(4)	PE(4)	PE-Hyp(4)	PE-O2(4)	p
Donor age	74±6	74±6	69±5	69±5	70±4	n.s
CIT	22.3±2.2	22.3±2.2	26.8±3.3	22.8±1.6	26.5±1.4	n.s
Remuzzi score	5	5	6	5.5	4.5	n.s.
pO_2 at T0	67±4	65±7	75±5	72±3	68±7	n.s
pO_2 at T1	176±48	282±29	106±37	366±83	710±39	<0.001
pCO_2 at T0	7±2.5	6.3±2.5	6.5±4.4	5.3±1.3	4.3±0.5	n.s
pCO_2 at T1	8.3±3.3	12±3.5	7±2.5	40±19.6	4.8±1	<0.001
Lactic acid at T0	0.3±0.2	0.2±0	4.3±5.9	0.7±0.8	2.2±0.6	n.s.
Lactic acid at T1	0.5±0.4	0.3±0.2	12.7±9.9	2.1±2.6	9.5±5.5	<0.001
pH at T0	6.99±0.05	7.03±0.04	6.96±0.08	6.98±0.01	7.04±0.03	n.s.
pH at T1	6.96±0.07	6.85±0.02	6.85±0.08	6.80±0.01	7±0.21	n.s

Data are expressed as mean (± standard deviation.)

CS: static cold storage. **Hyp:** static cold hyperbaric oxygenation with 1.5 atm. **PE**: hypothermic perfusion with arterial pressure of 25-30 mmHg. **PE-Hyp**: hypothermic perfusion in hyperbaric oxygenation with arterial pressure of 25-30 mmHg and with 1.5 atm. **PE-O2**: hypothermic oxygenated perfusion with arterial pressure of 25-30 mmHg and with 2-4 l of O_2 level maintaining pO_2 close to 700 mmHg. **CIT:** cold ischemia time. **TO**: before starting the treatment. **T1**: at the end of 3 hours of treatment.

Table 2. Demographic and clinical data for liver and kidney matching analyses.

	HOPE	SCS	P value
Liver Transplantation	*N = 10*	*N = 30*	
Donor Age (year)	77.5 (60-84)	75.5 (53-85)	0.396
Recipient Age (year)	57.5 (50-68)	60.5 (48-68)	0.331
Cold Ischemia Time (h)	7.1 (6.1-9.6)	7 (5.4-10)	0.528
MELD score	13 (7-16)	13.5 (7-20)	0.963
Previous abdominal surgery	5 (50%)	13 (43.3%)	0.681
Portal thrombosis	1 (10%)	3 (10%)	1
Hepatic Steatosis			
> 0% and < 10%	6 (60%)	18 (60%)	0.160
≥ 10% and ≤ 30%	4 (40%)	12 (60%)	
Kidney Transplantation	*N = 10*	*N = 30*	
Donor Age (year)	71.5 (60-78)	69.5 (59-79)	0.653
Recipient Age (year)	61 (50-65)	60.5 (48-68)	0.851
Cold Ischemia Time (h)	14.5 (10.8-22)	14 (8-21)	0.896
Karpinsky's score	4 (0-7)	3 (0-6)	0.105
Induction Immuno_Therapy			
Thymoglobuline	5 (50%)	4 (13.4%)	0.254
Simulect	5 (50%)	26 (86.6%)	
Type of Dialysis			
Peritoneal dialysis	4 (40%)	9 (30%)	0.492
Hemodialysis	6 (60%)	21 (70%)	
Dialysis Time (months)	47.3 (19.6-108)	47.3 (7.6-139.2)	0.931

The values are expressed as median (range) or number (percentage).

Table 3: Frequencies and percentages of the 9 histopathological variables evaluated in L-HOPE and L-SCS.

	HOPE	SCS	P value
Liver Transplantation	*N = 9*	*N = 16*	
Fibrosis (>2 Ishak)	2 (22.2%)	3 (18.8%)	0.524
Moderate portal inflammation	1 (11.1%)	4 (18.2%)	0.408
Lobular necrosis	2 (22.2%)	2 (11.1%)	0.352
Moderate/severe myointimal thickening	5 (55.6%)	7 (46.7%)	0.682
Biliocyte regressive changes	6 (66.7%)	9 (64.3%)	0.458
Ductular reaction	1 (11.1%)	3 (21.4%)	0.483
Cholestasis	2 (22.2%)	3 (15.8%)	0.527
Mean macrovesicular steatosis	3.1 ± 2.4	3.9±5.2	0.677
Mean microvesicular steatosis	8.11 ± 8.4	8.9±8.1	0.921

Table 4. Frequencies and percentages of the 13 histopathological variables evaluated in K-HOPE and K-SCS.

	HOPE	**SCS**	**P value**
Kidney Transplantation	*N = 11*	*N = 27*	
Glomerulosclerosis (Karpinski 1-2)	8 (72.7%)	11 (40.7%)	0.085
Tubular atrophy (Karpinski 1-2)	54.4%	77.8%	0.317
Interstitial fibrosis (Karpinski 1-2)	6 (63.6%)	21 (85.2%)	0.280
Vascular damage (Karpinski 2-3)	4 (36.4%)	5 (18.5%)	0.639
Mean Karpinski's score	3.6±2.0	3.4±1.3	0.639
Ischemic glomeruli	6 (54.5%)	6 (26.1%)	0.108
Mesangial matrix thickening	8 (72.7%)	12 (52.2%)	0.497
Glomerular microangiopathy	0 (0%)	3 (11.1%)	0.296
Acute tubular necrosis (diffuse)	4 (36.4%)	4 (17.4%)	0.271
Vascular changes: - sclerosis	8 (72.7%)	16 (69.6%)	0.591
- hyalinosis	3 (27.7%)	7 (30.4%)	
Isometric vacuolization of tubuli	6 (63.6%)	7 (30.4%)	0.060
Calcifications of tubuli	1 (9.1%)	0 (0%)	0.324
Interstitial inflammatory infiltrate	3 (27.7%)	4 (17.4%)	0.404

Table 5. Machine perfusion and biochemical data before (T0) and after (T1) hypothermic oxygenated perfusion (HOPE).

Organ	*Flow*	*Pressure*	*Resistence*	*Temperature (°C)*	*Time*	*pH T0*	*pCO_2 T0*	*pO_2 T0*	*Lac T0*	*pH T1*	*pCO_2 T1*	*pO_2 T1*	*Lac T1*
1_Kidney	55	30	0.54	4	60’	6.85	8	346	5.4	<6.8	9	647	9
2_Kidney	52.5	30	0.57	4	200’	6.88	7	204	4.5	6.83	7	706	11.7
3_Kidney	29	30	1.03	4	100’	<6.8	6	483	<2.7	<6.8	6	688	6.3
4_Kidney	46	30	0.65	4	360’	6.83	<6	779	<2.7	6.82	6	732	13.5
5_Kidney	30	30	1	4	240’	6.87	<6	112	<2.7	<6.8	6	784	6.3
6_Kidney	55	30	0.54	4	270’	6.86	<6	93	<2.7	6.82	8	778	12.6
7_Kidney	60	30	0.5	4	300’	7.03	7	185	3.6	6.94	8	718	11.7
8_Kidney	85	30	0.35	4	150’	7.17	<6	172	8.1	7.17	<6	723	22.5
9_Kidney	52	30	0.57	4	150’	6.82	<6	191	3.6	<6.8	6	741	10.8
10_Kidney	24	30	1.25	4	80’	<6.8	6	546	9.9	6.93	8	623	10
11_Kidney	77	30	0.32	4	205’	7.27	<6	132	<2.7	7.23	<6	675	12.6
1_Liver	25	5	0.2	4	75’	<6.8	6	78	3.6	<6.8	10	632	16.2
2_Liver	116	5	0.04	4	60’	6.88	8	155	6.3	6.84	8	688	10.8
3_Liver	110	5	0.04	4	170’	<6.8	9	190	8.1	<6.8	10	714	14.4
4_Liver	114	5	0.04	4	115’	<6.8	12	225	17.1	<6.8	11	582	24.3
5_Liver	100	5	0.05	4	165’	6.83	<6	105	2.7	6.83	6	706	9.9
6_Liver	109	5	0.04	4	130’	6.88	<6	123	<2.7	6.95	8	685	19
7_Liver	100.5	5	0.05	4	135’	6.86	<6	265	<2.7	6.83	10	605	9
8_Liver	106	5	0.05	4	210’	6.88	13	106	14.4	6.96	9	653	19.8
9_Liver	65	5	0.07	4	90’	7.23	<6	204	8.1	7.14	7	678	27
10_Liver	30	5	0.16	4	160	7.18	9	155	26.1	7.31	8	682	2.7

Table 6. Clinical outcomes of hypothermic oxygenated perfusion groups and control groups.

Kidney	*HOPE (N = 10)*	*Control (N = 30)*	*Odds ratio/Effect size (CI)*	*p value*
PNF, n (%)	0 (0%)	1 (3.3%)	0.9667 OR (0.3506-2.6656)	0.9478
DGF, n (%)	2 (20%)	12 (40%)	0.7500 OR (0.2501-2.2489)	0.6076
Creatinine at day 5 (mg/dL)	3.5 ± 2	4.1 ± 2.6	- 0.24 ES (-0.96 to 0.48)	0.4572
eGFR on discharge day (mL/min/1.73m²)	52.4 ± 25.01	44.43 ± 21.4	- 0.35 ES (-0.37 to 1.07)	0.3762
Hospital stay (days)	17 (12-30)	24 (11-60)	- 0.49 ES (-1.22 to 0.23)	0.0924
1-year graft survival	10 (100)	28 (93.3)	0.9333 OR (0.3377-2.5796)	0.8936
1-year patient survival	10 (100)	29 (96.6)	0.9667 OR (0.3506-2.6656)	0.9478
Liver	*HOPE (N = 10)*	*Control (N = 30)*	*Odds ratio/Effect size (CI)*	*p value*
PNF, n (%)	0 (0%)	2 (6.6%)	0.9633 OR (0.3377-2.5797)	0.894
EAD, n (%)	0 (0%)	7 (23.3%)	0.7667 OR (0.2734-2.1501)	0.613
Peak AST within 7 days (U/L)	**344 (166-1132)**	**637 (124-2100)**	**-0.82 EF (-1.55 to -0.08)**	**0.006**
Peak ALT within 7 days (U/L)	330 (122-1350)	601 (114-1837)	-0.52 EF (-1.24 to 0.20)	0.143
Bilirubin at day 7 (mg/dL)	3.14 ± 1.54	3.62 ± 3.22	-0.16 EF (-0.88 to 0.55)	0.538
INR at day 7	**1.17 (1.08-1.46)**	**1.24 (1.02-1.64)**	**-0.60 EF (-1.32 to 0.13)**	**0.043**
Hospital stay (days)	11.5 (7-29)	12.5 (7-109)	-0.28 EF (-1.00 to 0.44	0.235
1-year graft survival	10 (100%)	27 (90%)	0.9000 OR (0.3248-2.4937)	0.839
1-year recipient survival	10 (100%)	27 (90%)	0.9000 OR (0.3248-2.4937)	0.839

Data are expressed as mean (± Standard Deviation), median (range), or as absolute number (percentage).

Table 7. Post-operative complications according to Dindo-Clavien classification.

Dindo-Clavien grade	*K-HOPE (N = 10)*	*K-SCS (N = 30)*
I	0/10 (0%)	2/30 (6.7%)
II	1/10 (10%)	5/30 (16.7%)
IIIa	0/10 (0%)	1/30 (3.3%)
IIIb	1/10 (10%)	1/30 (3.3%)
IVa	2/10 (20%)	12/30 (40%)
IVb	0/10 (0%)	1/30 (3.3%)
V	0/10 (0%)	0/30 (0%)
Total	4/10 (40%)	22/30 (73.3%)

	L-HOPE (N = 10)	*L-SCS (N = 30)*
I	1/10 (10%)	4/30 (13.3%)
II	5/10 (50%)	10/30 (33.3%)
IIIa	1/10 (10%)	2/30 (6.7%)
IIIb	0/10 (0%)	1/30 (3.3%)
IVa	0/10 (0%)	3/30 (10%)
IVb	0/10 (0%)	0/30 (0%)
V	0/10 (0%)	1/30 (3.3%)
Total	7/10 (70%)	21/30 (70%)

7. References

1. Centro Nazionale Trapianti. Available at: http://www.trapianti.salute.gov.it/.
2. Deshpande R, Heaton N. Can non-heart-beating donors replace cadaveric heart-beating liver donors? J Hepatol. 2006; 45: 499-503.
3. Orman ES, Mayorga ME, Wheeler S, et al. Declining liver graft quality threatens the future of liver transplantation in the United States. Liver Transpl 2015; 21: 1040-50.
4. Ravaioli M, Grazi GL, Cescon M, et al Liver transplantations with donors aged 60 years and above: the low liver damage strategy. Transpl Int. 2009; 22: 423-33.
5. Bertuzzo VR, Cescon M, Odaldi F, et al. Actual Risk of Using Very Aged Donors for Unselected Liver Transplant Candidates: A European Single-center Experience in the MELD Era. Ann Surg. 2017; 265: 388-96.
6. Merion R, Ashby VB, Wolfe RA et al. Deceased-donor characteristics and the survival benefit of kidney transplantation. JAMA 2005; 294: 2726-33.
7. Ravaioli M, Grazi GL, Dazzi A, et al. Survival benefit after liver transplantation: a single European center experience. Transplantation. 2009; 88: 826-34.
8. Durand F, Ren JF, Alkofer B, et al. Report of the Paris Consensus Meeting on Expanded Criteria Donors in Liver Tranplantation. Liver Transplantation 2008; 14:1694-707.
9. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004; 364: 1814-27.

10. Salehi S, Tran K, Grayson WL. Advances in Perfusion Systems for Solid Organ Preservation. Yale J Biol Med. 2018; 91: 301-12.

11. Port FK, Bragg-Gresham JL, Metzger RA, et al. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. Transplantation.2002; 74: 1281-6.

12. Bruzzone P, Giannarelli D, Adam R; European Liver and Intestine Transplant Association; European Liver Transplant Registry. A preliminary European Liver and Intestine Transplant Association-European Liver Transplant Registry study on informed recipient consent and extended criteria liver donation. Transplant Proc. 2013; 45: 2613-5.

13. Attia M, Silva M A, Mirza D.F. The marginal liver donor – an update. Transplant International. 2008; 21: 713–724.

14. Karpinski J, Lajoie G, Cattran D, et al. Outcome of kidney transplantation from high-risk donors is determined by both structure and function. Transplantation. 1999; 67: 1162-7.

15. Kayler LK, Magliocca J, Zendejas I, et al. Impact of cold ischemia time on graft survival among ECD transplant recipients: a paired kidney analysis. Am J Transplant. 2011; 11: 2647-56.

16. Loinaz C, Gonzalez EM. Marginal donors in liver transplantation. Hepatogastroenterology 2000; 47:256-63.

17. Saidi RF. Change in Pattern of Organ Donation and Utilization in US. Int J Organ Transplant Med 2012; 3:149-56.

18. Angelico M. Donor liver steatosis and graft selection for liver transplantation: a short review. Eur Rev Med Pharmacol Sci 2005; 9: 295-7.
19. Verran D, Kusyk T, Painter D, et al. Clinical experience gained from the use of 120 steatotic donor livers for orthotopic liver transplantation. Liver Transplant 2003; 9: 500-5.
20. Imber CJ, St Peter SD, Handa A, Friend PJ. Hepatic steatosis and its relationship to transplantation. Liver Transplant 2002; 8: 415-23.
21. Remuzzi G, Grinyo J, Ruggenenti P, et al. Early experience with dual kidney transplantation in adults using expanded donor criteria. J Am Soc Nephrol 1999; 10: 2591-8.
22. Remuzzi G1, Cravedi P, Perna A, et al; Dual Kidney Transplant Group. Long-term outcome of renal transplantation from older donors. N Engl J Med. 2006; 354: 343-52.
23. Lee and Martin J. Mangino. Preservation methods for kidney and liver. Organogenesis 2009; 5: 105-12.
24. Southard JH, Belzer FO. Organ preservation. Annu Rev Med. 1995; 46: 235–47.
25. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation: Initial Perfusion and 30 Hours' Ice Storage. Lancet. 1969; 294: 1219–22.
26. Ploeg RJ, van Bockel JH, Langendijk PT, et al. Effect of preservation solution on results of cadaveric kidney transplantation. Lancet. 1992;340: 129–37.
27. Pedotti P, Cardillo M, Rigotti P, et al. A Comparative prospective study of two available solutions for kidney and liver preservation. Transplantation. 2004; 77: 1540–5.

28. Lindbergh CA. An apparatus for the culture of whole organs. Journal of Experimental Medicine, 1935

29. Belzer FO, Ashby BS, Gulyassy PF, Powell M. Successful seventeen-hour preservation and transplantation of human-cadaver kidney. N Engl J Med, 1968; 278: 608–10.

30. Starzl TE. Donor hepatectomy and liver preservation-ex vivo perfusion. In: Starzl TE, editor. Experience in Hepatic Transplantation. Philadelphia, PA: W.B. Saunders Company, 1969; 58–64.

31. Schlegel A, Rougemont O, Graf R, et al. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol 2013; 58: 278–86.

32. Jochmans I, Moers C, Smits JM, et al. Machine perfusion versus cold storage for the preservation of kidneys donated after cardiac death: A multicenter, randomized, controlled trial. Ann Surg 2010; 252: 756–64.

33. Wszola M, Kwiatkowski A, Diuwe P, et al. One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation. Transpl Int 2013; 26:1088–96.

34. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant 2010; 10: 372–81.

35. Weeder PD, van Rijn R, Porte RJ. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures: Rationale, current evidence and future directions. J Hepatol 2015; 63: 265–75.

36. Dutkowski P, Schlegel A, de Oliveira M, et al. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol 2014; 60: 765–72.

37. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: Better conditioning and repair? Transpl Int 2015; 28: 657–64.

38. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: A new paradigm? Transpl Int 2015; 28: 690–9.

39. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: The first clinical study. Am J Transplant 2013; 13: 1246-52.

40. Watson CJ, Kosmoliaptsis V, Rand LV, et al. Preimplant normothermic liver perfusion of a suboptimal liver donated after circulatory death. Am J Transplant 2016; 16: 353–7.

41. O'Callaghan JM, Morgan RD, Knight SR, Morris PJ. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcomes. Br J Surg 2013; 100: 991–1001.

42. Moers C, Smits JM, Maathuis MH, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 2009; 360: 7–19.

43. Hosgood SA, Mohamed IH, Bagul A, Nicholson ML. Hypothermic machine perfusion after static cold storage does not improve the preservation condition in an experimental porcine kidney model. Br J Surg 2011; 98: 943–950.

44. Treckmann J, Nagelschmidt M, Minor T, et al. Function and quality of kidneys after cold storage, machine perfusion, or retrograde oxygen persufflation: Results from a porcine autotransplantation model. Cryobiology 2009; 59: 19–23.

45. Hoyer DP, Gallinat A, Swoboda S, et al. Influence of oxygen concentration during hypothermic machine perfusion on porcine kidneys from donation after circulatory death. Transplantation 2014; 98: 944–950.

46. Gallinat A, Paul A, Efferz P, et al. Role of oxygenation in hypothermic machine perfusion of kidneys from heart beating donors. Transplantation 2012; 94: 809–813.

47. Hosgood SA, Nicholson HF, Nicholson ML. Oxygenated kidney preservation techniques. Transplantation 2012; 93: 455–459.

48. Schlegel A, Graf R, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) protects from biliary injury in a rodent model of DCD liver transplantation. J Hepatol 2013; 59:984–991.

49. Op den Dries S, Sutton ME, Karimian N, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One 2014; 9: e88521.

50. Dutkowski P, Polak W, Muiesan P, et al. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants: An international-matched case analysis. Ann Surg 2015; 262: 764–771.

51. Hosgood SA, Barlow AD, Hunter JP, Nicholson ML. Ex vivo normothermic perfusion for quality assessment of marginal donor kidney transplants. Br J Surg 2015; 102: 1433–1440.

52. Op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013; 13: 1327–35.

53. Ravikumar R, Jassem W, Mergental H, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016; 16: 1779-87.

54. Perera T, Mergental H, Stephenson B, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. Liver Transpl 2016; 22: 120–124.

55. Di Laudo M, Ravaioli M, La Manna G, et al. Combined liver-dual kidney transplant: Role in expanded donors. Liver Transpl. 2017; 23: 28-34.

56. Ravaioli M, Grande G, Di Gioia P, et al. Risk Avoidance and Liver Transplantation: A Single-center Experience in a National Network. Ann Surg. 2016; 264: 778-86.

57. Fiorentino M, Vasuri F, Ravaioli M, et al. Predictive value of frozen-section analysis in the histological assessment of steatosis before liver transplantation. Liver Transpl 2009; 15: 1821-5.

58. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology 1994; 20: 15-20.

59. Ishak K, Baptista A, Bianchi L et al. Histological grading andstaging of chronic hepatitis. J. Hepatol 1995; 22: 696–9.

60. Gaber LW, Gaber AO, Tolley EA, Hathaway DK. Prediction by postrevascularization biopsies of cadaveric kidney allografts of rejection, graft loss, and preservation nephropathy. Transplantation 1992; 53: 1219-25.

61. Nadasdy T, Laszik Z, Blick KE, et al. Human acute tubular necrosis: a lectin and immunohistochemical study. Hum Pathol 1995; 26: 230-9.

62. Ravaioli M, Baldassare M, Vasuri F, et al. Strategies to Restore Adenosine Triphosphate (ATP) Level after More than 20 Hours of Cold Ischemia Time in Human Marginal Kidney Grafts. Ann Transplant. 2018 Jan 12; 23: 34-44.

63. Stieber AC, Marsh JW Jr, Starzl TE. Preservation of the retrohepatic vena cava during recipient hepatectomy for orthotopic transplantation of the liver. Surg Gynecol Obstet 1989; 168: 542-4.

64. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. Am J Transplant 2009; 9 Suppl 3: S1-155.

65. Martin P, Di Martini A, Feng S, et al. Evaluation for liver transplantation in adults: 2013 practice guideline by the American Association for the Study of Liver Diseases and the American Society of Transplantation. Hepatology 2014; 59: 1144–65.

66. Olthoff KM, Kulik L, Samstein B, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transpl. 2010; 16: 943-949.

67. Humar A, Ramcharan T, Kandaswamy R, et al. Risk factors for slow graft function after kidney transplants: a multivariate analysis. Clin Transplant 2002; 16: 425-429.

68. Ravaioli M, Grazi GL, Ercolani G, et al. Liver allocation for hepatocellular carcinoma: a European Center policy in the pre-MELD era. Transplantation 2006; 81: 525-30.

69. Dindo D, Demartines N, Clavien PA. Classification of surgical complications- a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004; 240: 205–213.

70. Wijermars LG, Schaapherder AF, de Vries DK, et al. Defective postreperfusion metabolic recovery directly associates with incident delayed graft function. Kidney Int 2016; 90: 181-91.

71. Bosco G, Yang ZJ, Nandi J, et al. Effects of hyperbaric oxygen on glucose, lactate,

glycerol and anti-oxidant enzymes in the skeletal muscle of rats during ischemia and reperfusion. Clin Exp Pharmacol Physiol 2007; 34: 70-76.

72. Nørlinger TS, Nielsen PM, Qi H, et al. Hyperbaric oxygen therapy reduces renal lactate production. Physiol Rep 2017; 5: e13217.
73. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol 2006; 70: 1469-80.
74. Coulet F, Nadaud S, Agrapart M, Soubrier F. Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. J Biol Chem 2003; 278: 46230-40.
75. Schlegel A, Kron P, Dutkowski P. Hypothermic machine perfusion in liver transplantation. Curr Opin Organ Transplant. 2016; 21: 308-14.
76. Schlegel A, Muller X, Dutkowski P. Hypothermic Machine Preservation of the Liver: State of the Art. Curr Transplant Rep. 2018; 5: 93-102.
77. Kron P, Schlegel A, Mancina L, et al P. Hypothermic oxygenated perfusion (HOPE) for fatty liver grafts in rats and humans. J Hepatol. 2017; 68: 82-91.
78. Ravaioli M, De Pace V, Comai G, et al. Successful Dual Kidney Transplantation After Hypothermic Oxygenated Perfusion of Discarded Human Kidneys. Am J Case Rep. 2017 Sep 20;18:1009-13.
79. Nasralla D, Coussios CC, Mergental H, et al; Consortium for Organ Preservation in Europe. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018; 557:50-56.