UNIVERSITÀ DEGLI STUDI DI TORINO

Doctoral School in Life and Health Sciences

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EXPERIMENTAL MODEL OF NORMOTHERMAL PERFUSED RAT LIVER RECONDITIONED WITH HEPATIC STEM CELL DERIVED SUBSTANCES

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DOCTORAL THESIS

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XXXII Cycle

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1. LIVER TRANSPLANTATION

1.1 History

Liver transplantation is the replacement of a deceased liver with the healthy liver from another person. It is a life-saving treatment for end-stage liver disease and acute liver failure.

There are different kind of liver transplantation techniques: the most common is *Orthotopic Liver Transplantation* - OLT, in which the native liver is removed and replaced by the donor graft in the same anatomic position as the original liver. The *Living Donor Liver Transplantation* – LDLT consists in transplantation of a part of a living donor's liver, while the *split liver* is performed by splitting the liver of a deceased donor in order to obtain two functionally autonomous parts of the organ to transplant to two different recipients (usually an adult and a pediatric recipient). The indications to this kind of transplantation is given depending on the recipient's characteristics and clinical history but also on the graft qualities.

In 1952 for the first time transplantation procedure was described in an animal model by the Italian Vittorio Staudacher[1] while the first attempted human liver transplant was performed in Denver in 1963 by Thomas Starzl[2], although the pediatric patient died intraoperatively due to uncontrolled bleeding. Multiple subsequent attempts by the same surgeon were unsuccessful until 1967, when the same Starzl's équipe transplanted a 19 month old girl with hepatoblastoma who was able to survive for over 1 year. From 1967 to 1977 about 200 transplants were performed, all burdened by an hight mortality due to technical difficulties and the lack of an effective immunosuppression. In the 80's the introduction of ciclosporin[4] by sir Roy Calne and the preservation solution of Wisconsin University[5] markedly improved patients outcomes and signed the beginning of a new era in the transplantation history. In the following years many advances in the immunosuppression field and the improvement of the surgical technique trasformed liver transplantation from an experimental treatment to a standardized and solid

procedure. Today liver transplantation is an effective treatment, characterized by optimal results both in terms of survival and quality of live.

1.2 Liver transplant indications in adults

The most common indications for liver transplant are the following:

- Alcoholic Cirrhosis
- Viral Cirrhosis (mainly HBV and HCV)
- Hepatocellular Carcinoma - HCC
- Primary Biliary Cirrhosis (PBC)
- Primary Sclerosing Cholangitis (PSC)
- Autoimmune liver disease
- Idiopatic liver disease
- Wilson's disease
- Hemochromatosis
- alpha1-antitrypsin deficiency
- Amyloidosis
- Budd-Chiari Syndrome
- Polycystic liver disease
- Others: Caroli's disease, familiar hypercholesterolaemia, fulminant hepatitis, Crigler-Najar Syndrome, Cystic Fibrosis.

As showed in Figure 1, the most common disease indication for liver transplantation is viral cirrhosis, still today, although in the following years a marked decrease is expected for this indication, thanks to the effectiveness of the vaccine against HBV ant the new therapies for HCV infection.

Almost one patient in five is affected by an hepatocellular carcinoma at the moment of transplantation (figure 1); the indication for HCC is increasing in the last years (figure 2).

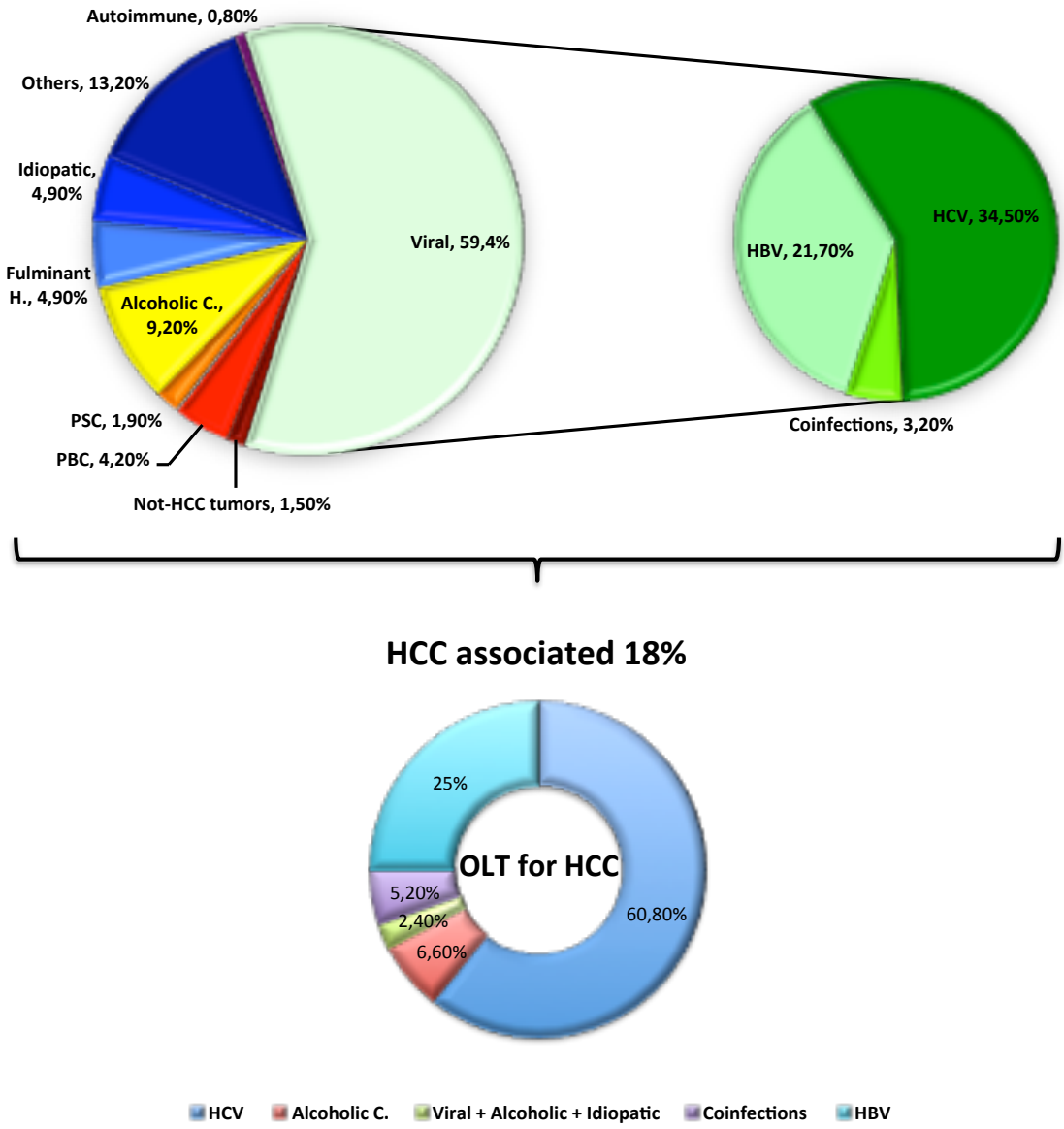


Figure 1. Indications to liver trasplant in adults for end-stage liver disease and for HCC in Italy. Each disease is presented with the percentage of casis on the total. [6]

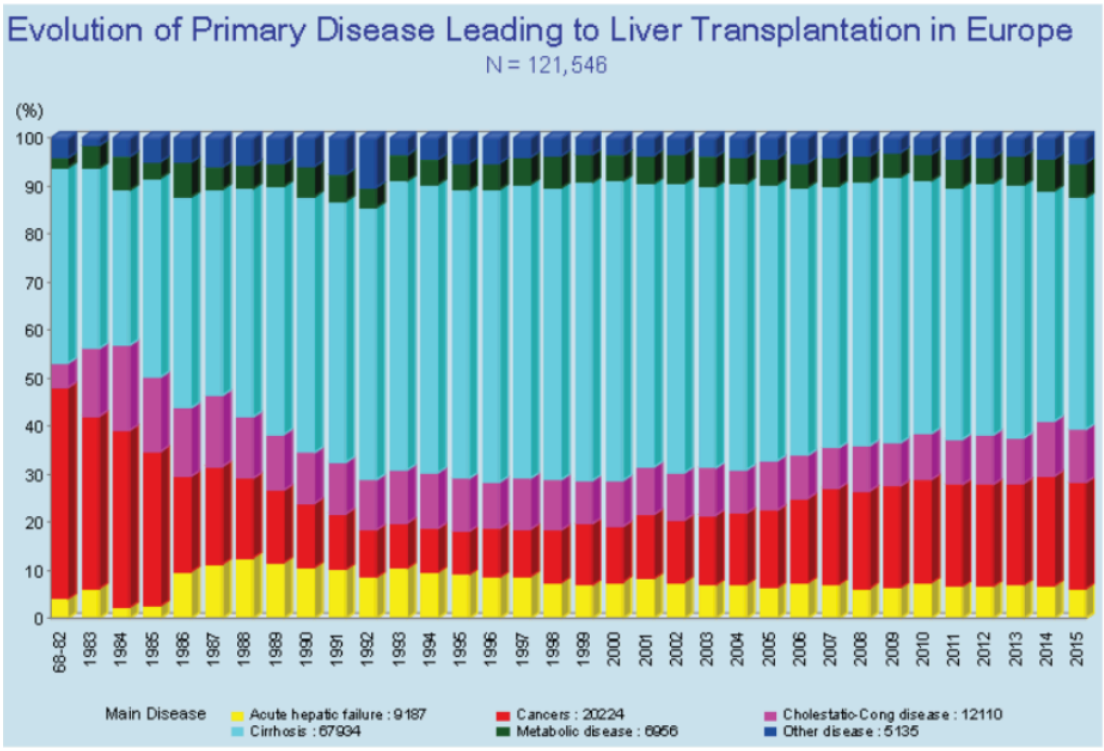


Figure 2. Evolution of indications to OLT from 1968 to 2015. Data from *European Liver Transplant Registry*

1.3 Waiting list, organs eligibility and allocation criteria

Before being placed on a waiting list for liver transplant, the patient affected by a liver disease should not present controindications and should meet particular minimum requirements. The process begins with the proposal by the family doctor, or the Gastroenterologist or by an infectiologist doctor. Then the transplantation unit provides for the assessment of patient suitability, in accordance with objective, shared and documented principles. In particular the candidate will be evaluated through many diagnostic and stadiative investigations, and his/her level of risk will be quantified using the two most common classifications: the Model for End-Stage Liver Disease Score (MELD) and the Child-Turcotte-Pugh Score (CTP).

MELD Score

MELD score is a score system used as a predictor of short term mortality (3 months), the variables for calculating the score are INR, bilirubin and creatinine (figure 3).[7] The results can vary from 5 to 40 points, to be included on the waiting list a MELD

score ≥ 15 is required (or <15 if the patient is affected by HCC meeting Milano's Criteria).

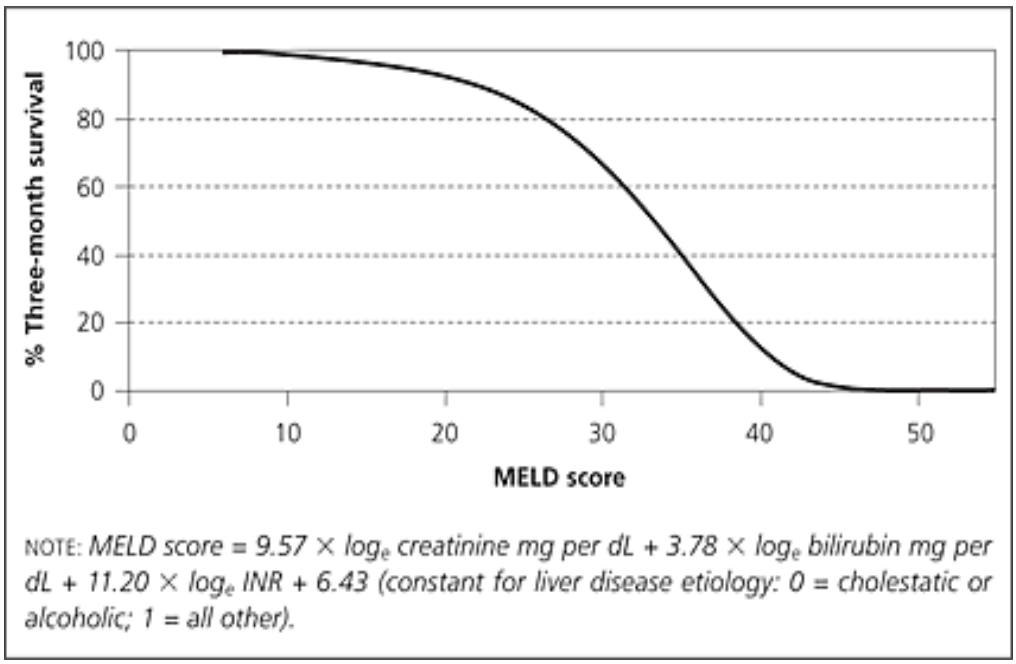


Figure 3. 3 month survival related with MELD score

CTP Score

The CTP classification considers the serum albumine, bilirubin, PT (INR), the presence of ascites and the severity of encephalopathy in order to stratify the patients in 3 levels: A, B and C (table 1).[8]

PARAMETERS	1 point	2 points	3 points
Albumin (g/dL)	> 3,5	2,8 - 3,5	< 2,8
Bilirubine (mg/dL)	< 2	2 - 3	> 3
INR	< 1,7	1,7 - 2,3	> 2,3
Ascites	None	Low - Mild	Severe
Encephalopathy	None	Grade 1-2	Grade 3-4
SCORE	**CLASS**	**1yOS**	**2yOS**
5-6	A	100%	85%
7-9	B	81%	57%
10-15	C	45%	35%

Table 1. CTP SCORE: the scoring system is in the highest part, in the lowest part the table shows the overall survival after one and two year stratified for CTP class.

The conditions listed above are applied in general for patients suffering from end-stage cirrhosis, moreover there are some special criteria for specific diseases.

- HCC: the candidates for liver transplantation with HCC must meet Milano's Criteria[9] which means: a) a single nodule ≤5 cm or ≤3 nodes ≤3 cm each, b) absence of macroscopic vascular invasion of main intrahepatic or extrahepatic venous branch, c) absence of extrahepatic disease. Fulfilment of these criteria guarantees a survival of 85% at 4 years, a low recurrence rate and a decreased risk of complications.
- Alcoholic cirrhosis: an accurate evaluation of the recipient is mandatory; it is required to demonstrate six months of sobriety before the patients are allowed to be registered on the waiting list.
- Cholestatic cronic diseases such as PSC and PBC: liver transplantation is recommended only if the probability of death in 1 year is ≥ 10% and/or in case of *Mayo Risk Model* equal to 6-7.[10] The patient suffering from PSC can be put on the waiting list even in the absence of liver impairment, just for his increased risk of developing cholangiocarcinoma.[11]

Concerning the contraindication for waiting list inclusion, these can be cliassified as absolute, relative and temporary:

Absolute contraindications:

- Active extrahepatic malignant neoplasia or previous neoplasia with follow-up < 5 years
- Full-blown AIDS
- Advanced stage cardiopathy and/or pulmonary disease
- Severe and irreversible neurological disorders
- Not resectable cholangiocarcinoma
- Multi-organ failure
- Severe psychiatric disorders
- Addiction or current drugs consumption or active intake of alcohol

Relative contraindications

- Pulmonary hypertension
- HIV+
- Thrombosis of the portal circle
- Previous extensive surgical operations on abdominal organs

Temporary contraindications

- Infections not involving hepatobiliary system (resolution of the infection is needed)
- HBV-DNA Positivity (antiviral therapy is necessary until the achievement of negativity or a low viremic level)

The patient that, after accurate multidisciplinary evaluation, is considered eligible for liver transplantation, will be put on the waiting list and stratified to define a level of priority. Within the most important criteria for organ allocation it is worth mentioning the following:

- severity of the illness with regard to the clinical status according to UNOS classification (table 2) and MELD score (table 1)
- date of placement on the waiting list
- compatibility between graft dimension and recipient size
- blood type match
- compatibility between recipient's and donor's age class (<20 yr, 20-55 yr, >55 yr)
- Possibility of allocation of "marginal" grafts to patients with advanced disease (cfr. chapter 2)

Status I	Acute liver failure
Status 2A	Chronic liver failure with acute worsening of clinical conditions and life expectancy shorter than 7 days without transplantation, CTP score>10 and at least one of the following conditions: - hepato-renal syndrome - recent spontaneous bacterial peritonitis (SBP) - ascites and hydrothorax refractory to treatment - portosystemic encephalopathy (PSE) grade III or IV refractory to medical treatment If the patient survives more than 7 days, he will be reclassified as 2B

Status 2B	CTP score >10, or CTP >7 with at least one of the following conditions: - hepato-renal syndrome - recent spontaneous bacterial peritonitis (SBP) - ascites and hydrothorax refractory to treatment - complete portal thrombosis - monofocal HCC in progress or multifocal HCC - "bridge" procedure (TIPS-Transjugular intrahepatic portosystemic stent shunting, treatment with lamivudine if at the beginning CTP was >10)
Status 3	CTP >7, or CTP <7 if associated with hepatic metabolic disease or cholestatic disease (Mayo score > 6/7)
Status 4	Patient registered on the waiting list for transplantation but temporarily suspended

Table 2. Priority criteria (modified NITp-North Italy Transplant program)[6]

Allocation criteria are established and managed at regional level by CRT (Regional Transplantation Center) using decision-making algorithms disclosed to CNT (National Transplantation Center) dictated by shared and scientifically based common principles, that can be documented to anyone who make a request to the CNT. The CNT is responsible to supervise the proper application of the algorithms.

In particular there are five way of allocation of available organs, defined by the Agreement of Conferenza permanente per i rapporti tra lo Stato, le Regioni e le Province Autonome di Trento e Bolzano on 23/09/2004:

- Scheduled allocation: every tranplantation center choose the candidate following its allocation criteria, taking into account primarily the blood-type match, the clinical status of the recipient, the anthropometric data and the time of waiting on the list (on equal clinical conditions, the priority is given depending on the waiting time).
- Emergency allocation: every transplantation center can notify an urgent request of graft to the corresponding CRT. CRT spreads the request at a national/international level. The regional transplantation center of the suitable donor will accept and make the graft available. If there are more than one emergency request, the chronological priority will be respected.
- Anticipated allocation: every transplantation center can submit to the corresponding CRT a request of an anticipated liver. That request can be extended nationally. There is no obligation for the transplantation center owner

of the available liver of giving the graft, but in case of rejection a written justification should be provided by the center.

- Restitution allocation: it includes the allocations related with the obligation to return all the graft received as anticipated or emergency allocation.
- Surplus allocation: in case of excess organs, the CRT ensure the allocation in its area or, if necessary, at national level. In this case there is no obligation to return the graft.

1.5 Operative technique

Liver transplantation is defined as orthotopic allotransplantation. Allotransplantation because the donor and the recipient belong to the same species and have similar, but not identical, genetic pool. Orthotopic since the graft is placed in the same position of the native organ, respecting the natural anatomical relationship.

The surgical procedure can be performed following two main techniques:

In the classical technique the inferior vena cava (IVC) is clamped suprahepatic and subhepatic and the tract of cava in between the two clamps is removed "en block" with the liver. To perform this technique a temporary by-pass for extracorporeal circulation is needed.

The alternative "piggy-back" technique, today the most performed, allows to avoid the venous by-pass, preserving the inferior IVC of the patient and consequently the venous return during the surgical operation. The IVC in clamped tangentially and anastomosis is performed between the supra-hepatic part of donor IVC and the common orifice of all three hepatic veins.

The first phase of liver transplantation (pre-anhepatic phase) consists in the total hepatectomy in the recipient. The procedure includes the section of the hepatic ligaments allowing the complete mobilization of the liver and the isolation of the hepatic pedicle structures. In the "piggy-back" technique the IVC is left in the natural place and the surgeon performs the dissection and section of the suprahepatic veins. On the contrary, in the classic technique the vena cava is isolated and removed together with the native liver.

The anhepatic phase starts from the total hepatectomy and ends at the moment of graft reperfusion, after confectioning the two venous vascular anastomosis. This is a critical phase, characterized by haemodynamic and metabolic alterations consequence of the temporary absence of the liver function and the sudden decline of the venous return (expecially in the classic technique). The deficit of liver function during this phase can be compensated monitoring and adjusting any possible electrolyte imbalance and acidosis. Moreover the venous return's impairment causes a reflex decrease of cardiac output, with the risk of developing an acute renal failure. In the classic technique the IVC is clamped and cutted in two points, respectively subdiaphragmatic and above renal veins. Therefore the anastomosis are performed following the sequence: 1) supra-hepatic IVC anastomosis, 2) sub-hepatic IVC anastomosis, 3) portal anastomosis. In order to limit the heamodinamic umbalance caused by the IVC clamping, sometimes a veno-venous shunt is necessary: portal vein (PV) and IVC are cannulated and the drained blood is reinfused in the axillary vein by means of an extracorporeal pump. In the piggy-back technique instead, the VC is clamped only partially and it does not imply the venous flow interruption. After removing the native liver and placing the graft in the orthotopic site, first the VC anastomosis and then the PV anastomosis are performed. From the moment in which the graft is settled in the abdominal cavity of the patient the warm ischemia time (WIT) begins, consequently the anastomosis should be made as fast as possible to limit the ischemia injury for the liver. At the end of this phase, the leakage test is carried out followed by the sequential declamping. At the moment of reperfusion, the WIT finishes. If the perfusion in the parenchima is omogeneous and there are no leakages, the subsequent step is the hepatic artery (HA) anastomosis and the bile duct anastomosis confection. The biliary tract can be reconstructed by a end to end bile duct anastomosis or by means of a Roux-en-Y hepato-jejunal anastomosis.

After declamping the post-reperfusion syndrome phase may occur. It is characterized by a combination of metabolic and haemodynamic alterations triggered by the systemic overload of toxins and cytokine released from the graft, from the splanchnic circle and from the caudal part of the patient's body. During this period a careful anaesthesiological monitoring is necessary.

Finally the liver transplantation procedure ends with drainages placement and closure of the surgical wound following anatomical planes.

2. NEW DONATION CRITERIA

2.1 Standard donation

Organs donation is a voluntary, conscious, free and anonymous act at the basis of every transplantation activity. In Italy the rules that regulate organ donation are expressed mainly in the Law n° 91 in the 1st April 1999 and in the Decree from the Ministry of Health in the 8th April 2000: every adult citizen can express his/her consent (or dissent) regard organ and tissue donation, and this decision can be manifested by means of one of the following procedures:

- Signing of the proper form available at the local health agency;
- Reporting at the Registry Office at the time of issue or renewal of identity card;
- Filling the specific blue card provided by the Ministry of Health (or similar card distributed by trade associations);
- Compilation of the holographic will of AIDO (Italian Association of Organ Donors);
- Declaration on a white sheet inclusive of date and signature.

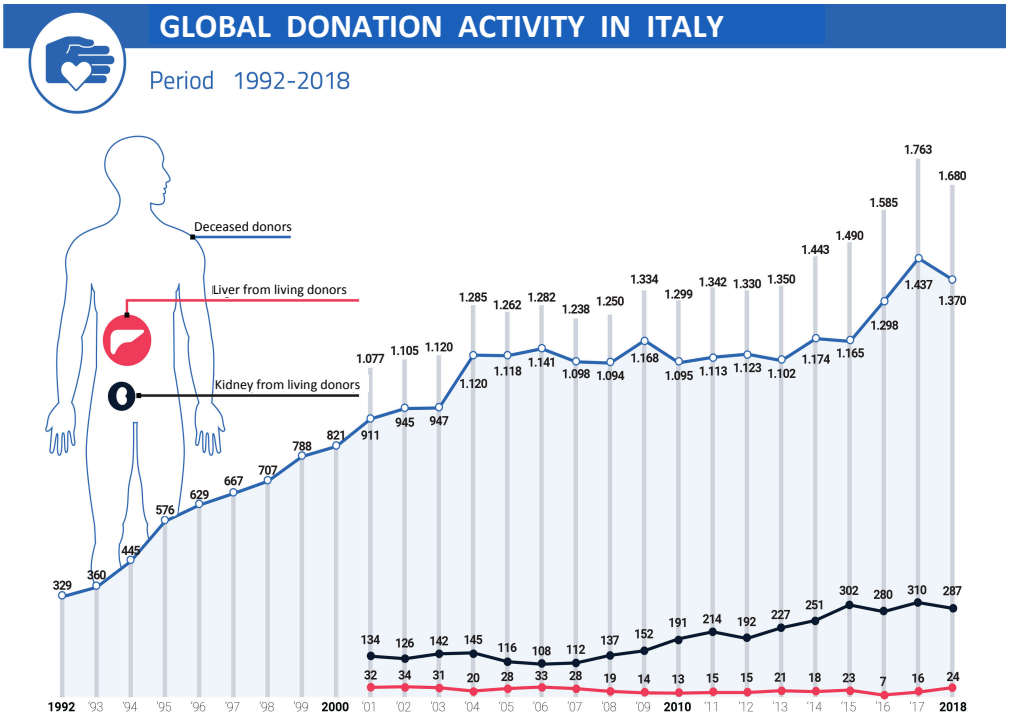


Figure 4. Donation activity in Italy from 1992 to 2018. Font: annual report of CNT

In absence of a written declaration in life, the relatives will be consulted about the intention on organ donation of the deceased. If they deny the consent the procurement can not be performed. In case of a minor donor those responsible for the decision are the parents.

Italy is in third position in Europe for number of donations between countries with a population greater than 40 milions, following Spain and France[13]. Italian trend is increasing and in 2018 there have been 1370 donations and over 4 milions of declarations of will registered in the transplantation informative system (figure 4 and 6).[12]

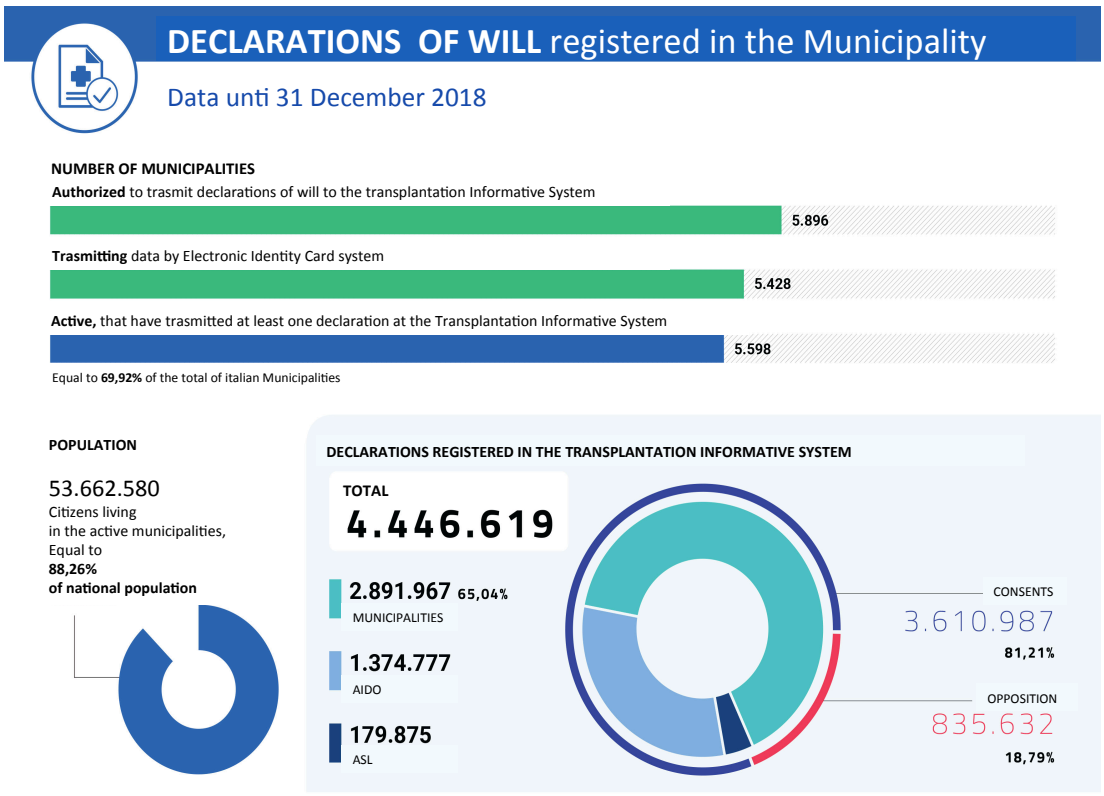


Figure 5. Declaration registered in the Transplantation Informative System until 31 December 2018. Font: Annual report of CNT.[12]

The evaluative procedures that lead to organ procurement for transplantation starts with verification of the donor's death. Death is defined as the definitive cessation of all brain functions. (Law n°578 - 29 December 1993) and it could be established through two criteria:

a) Cardiac death

Death for cardio-circulatory arrest occurs as a consequence of the cessation of cardiac and respiratory activity for a sufficient period of time to cause the irreversible cessation of the brain functions. The Italian law establishes the lenght of time as 20 minutes and the necessity of continuous flat electrocardiogram registration. In many other countries this period is shorter (usually 5-10 minutes). This fact makes more difficult in Italy than in other countries the transplantation of organs from Donors after Circulatory Death (DCD), since these organs inevitably suffers an extremely prolonged Warm Ischemia Time (WIT) (cfr. chapter 3).

b) Brain death

Brain death occurs in case of irreversible brain injuries in patients receiving intensive care. The verification of brain death requires an observational period of 6 hours, at least. During this time a commettee of three different specialists (usually a coroner, an anesthesiologist and a neurologist or a neuropathophysiologist) verifies:

- Absence of brain stem reflexes;
- Absence of any spontaneous respiratory efforts during apnea test;
- Absence of brain electrical activity evaluated by means of electroencephalography (EEG);

It is also appropriate to evaluate the absence of cerebral blood flow using angioTC or echodopller in every case of doubt. It is mandatory in children under 1 year, in cases in which central nervous system suppressors can not be suspended or where EEG is impossible to perform.

Once the death is confirmed, the consent for organ donation have to be verified according to the procedure described before. In case of acceptance for donation the following step is the analysis of donor's suitability. This procedure's main goal is to minimize transplantations complications. It consists in a multidisciplinary multi-phase evaluation that involves anesthesiologists, local coordinators and the Regional Transplantation Center. The process includes mandatorily the following steps:

- Medical history of the donor: in order to identify the potential presence of previous or in place infectious deseases and tumors. The collection of anamnestic

data should be carried out by interviewing all the available fonts of information (family doctor, relatives, acquaitances, hospital staff, etc).

- External physical examination: It's for identifying signs attributable to trasmissible deseases (scars, mucocutaneous lesions, in particular pigmented lesions, tatoo, piercing, jaundice and rashes). Furthermore palpation of superficial limph nodes, breast, testicles and thyroid is performed. If the donor is more than 50 years also the digital rectal examination is required.
- Standard biochemical tests: blood cell count, platelets count, serum creatinine, azotemia, electrolytes (Na, K, Ca, Cl), total serum proteins, albumine, troponin, glycemia, cytolysis and cholestasis enzymes, bilirubine, amylase, lipase, coagulation, complete urine analysis, standard blood-gas at 100% of Oxygen, PSA (Prostatic Serum Antigen) (total/free) in men over 50's, β chorionic gonadotrophin (βHCG).
- Serological tests for HIV, HCV, HBV, HDV, CMV, HSV, EBV, VZV, syphilis, toxoplasma and additional test if there is the suspect of a specific diseases
- Culture test on blood, urine, sputum, rectal swab.
- Instrumental tests: electrocardiogram, echocardiogram, thorax x-ray, abdomen US scan.

On the basis of clincal and anamnestic data the donor is defined according to his risk profile as a "eligible standard risk donor", "eligible NOT standard risk donor" (marginal donor) or "NOT eligible donor".[14]

2.2 Extended criteria donors

Liver transplantation is today the only effective therapy for end-stage liver diseases and it guarantees a mean survival at 1 year of 86,5%.[12] However its main limit is the impossibility to satisfy the increasing request of organs. As shown by the annual report of the Italian Transplantation National Center, despite the intense activity of the italian transplantation centers (1246 liver transplantation only in 2018), there are still 960 patients on the waiting list at 31/12/2018 and the mean waiting time is 1,6 years (figure 6).[12] These data show the large gap between the supply and the demand of organs. It is evident the urgent need of finding new strategies in order to increase the number of suitable livers for transplantation.

At the moment the possible approaches are the Living Donor Liver Transplantation - LDLT), the split-liver and the transplantation of marginal organs or organs from extended criteria donors.

The procedure of LDLT consists in a partial hepatectomy performed in a volunteer donor and the transplantation of the removed part on a recipient that meets all the inclusion criteria of the standard transplantation list.[15] Developing of this thecnique was possible thanks to the hight regenerative potential of the liver and the improvements of surgical thecniques for liver resection.[16]

In a split-liver transplantation the donor's liver is divided in two portions, that will be allocated to two different recipients, usually an adult and a pediatric patient. In particular the right lobe is transplanted in an adult recipient and the left lobe in a pediatric patient or in an adult with a tiny physical constitution (<55 kg).[17]

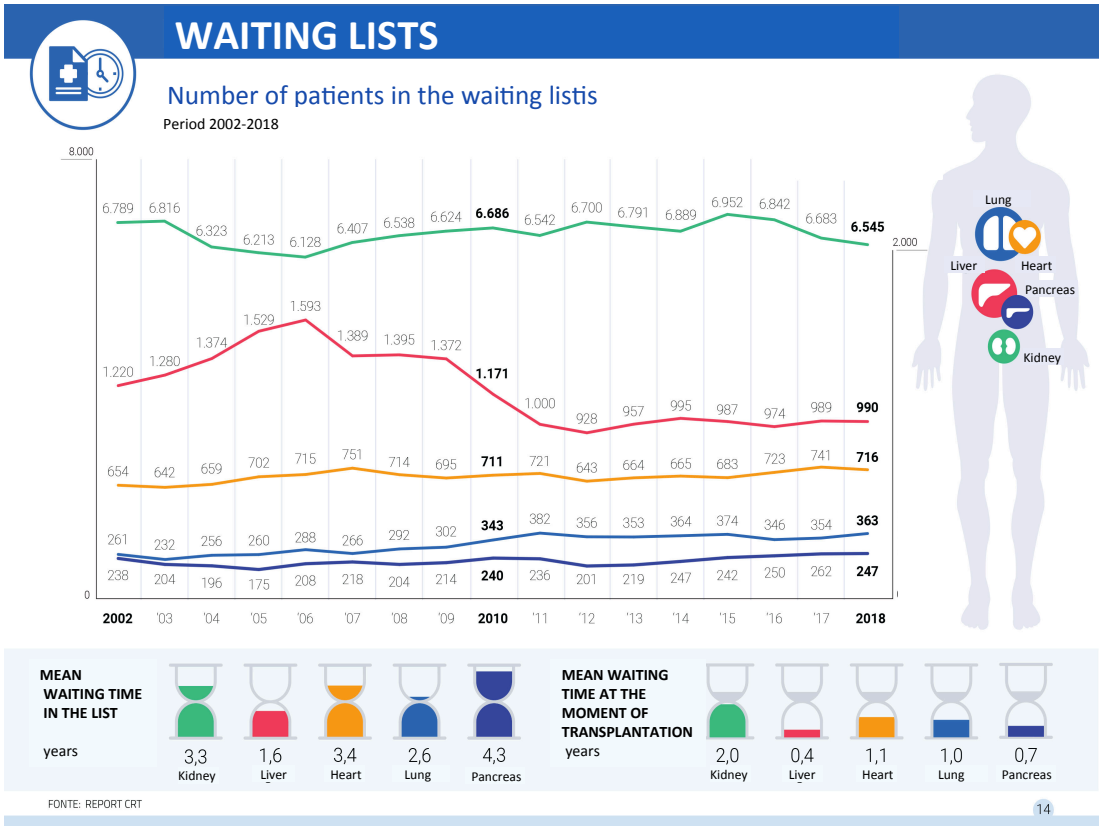


Figure 6. Number of patients in the waiting list and mean time of waiting. Font: annual report of CNT activity.[12]

The two described techniques have assured during the past years a major increase of accessibility to transplantation (expecially for pediatric patients) and

results in term of survival absolutely comparable to standard procedures; nevertheless they can be applied only in a small percentage of transplantations: in 2018 LDLT and *split-liver* transplantations were only 7% of the total.[12]

Another promising option to increase donor's pool is represented by the extension of the selection parameters for organs allocated for transplantation. This can happen by accepting also the so called "marginal organs" (or sub-optimal organs) by the application of extended criteria. For this reason this kind of organ donors are called Extended Criteria Donors (ECD). Although an universal and common definition of marginal graft does not exist, usually all the organs affected by an increased incidence of post-transplant failure or an higher risk of deseases transmission are included in this category. (table 3).[18,19]

Risk of organ failure	Risk of diseases transmission
Macrovescicolar steatosis > 25%	Anti-HCV +
Obese donors	Anti-Hbc+
Old donors (> 70 years)	HbsAg+
DCD	Donors affected by malignant neoplasia
Ipersodiemic donors	Donors carriers of a bacterial infection
Long stay in Intensive Care Unit	"High risk life style" donors

Table 3. Classification of the main categories of marginal donors.

ECD organs at risk of diseases trasmission

Concerning the risk of trasmission of viral infection there are specific protocols that limit the transplantation of graft from infected donors only to recipient already infected or owning a specific immunity:[20]

- A liver from anti-HCV positive donor can be transplanted on a recipient affected by HCV positive cirrhosis, after a careful selection, since the survival results are comparable to those reported in transplantation with anti-HCV negative donor. However the histological evaluation is crucial in every case in order to exclude the presence of fibrosis.

- Livers from anti-HBc positive donors can be used for HBsAg-positive recipient, with prophylactic schemes normally used in this kind of infection, and in anti-HBc positive/HBsAg negative recipient only in association with specific immunoglobuline prophylaxis for an unlimited period.
- According with the current legislation the transplantation from a HBsAg positive donor can be performed only on HbsAg positive recipients, prior specific consent and only in case of demostrated clinical urgency, following the instructions issued by the Ministry of Health (GU n°297 of the 23 december 2003).

About donor's bacterial infection, it is important to clarify that they do not represent an absolute contraindication for donation, provided that the agent responsible for the infection is known and an antibiogram is available. Naturally the organ to transplant should not to be site of infections such as in case of liver abscesses and cholangitis.[14]

If a malignant tumor is detected during the procurement or through the questions about the medical history of the donor, this must be investigated in order to define a prognosis. The mere presence of a neoplasm does not preclude the utilization of the organ absolutely, but a careful evaluation is mandatory in order to establish a risk/benefit balance for the recipient.[14]

ECD livers with an increased risk of organ dysfunction or non-function

A part of ECD livers is burdened by an higher incidence of complications, expecially at short term. Particularly, the two most used parameters to define the severity of short term complications are the "Primary Non Function" (PNF) and the "Early Allograft Disfunction" (EAD). PNF is defined as the functional failure of the graft not caused by HA thrombosis, biliary complications, recurrence of disease or acute rejection, requiring an urgent re-transplantation until 7 days to avoid the death of the patient.[21] According to Olthoff's Criteria[22] EAD is defined as the presence of one of the following laboratory values during the postoperative course: bilirubin ≥ 10 mg/dL on day 7, INR ≥ 1,6 on day 7 and AST or ALT> 2000 IU/L within the first 7 days after surgery. EAD, on the contrary of PNF, is a potentially reversible condition, but it is associated with an higher incidence of re-transplantation within 30

days, with a shorter graft sirvival and an increased 6 and 12 months mortality of the patient.

On the other hand, in the situation of donor's deficiency, the main pool of recruitment is represented by organs within this category, in particularly by livers from steatotic, old or DCD donors.[23]

Age of donors is increasing costantly compared with the past. In 1991 only 13% of donors was more than 50 years, today that percentage greatly exceeds 70%.[24] At the beginning it was thought that livers from over 50 donors were associated with a greater risk, but many studies demostrated that organs of donors from 50 to 70 years old are not related with an increase of complications.[25,26] However, as regards over 70 donors, there is evidence of worsening of outcomes,[27,28] possibly dued to a greater sensitivity to the ischemic damage.[29,30] On the other hand, the increased number of complications seems to be related with the simultaneous pathological conditions of the donor rather than the age itself. For this reason, in case of very old donors, a careful analysis of associated risk factors is mandatory.[31,32]

Liver steatosis, that is the accumulation of triglycerides in hepatocytes, represent another detrimental condition for graft's quality and for the outcome of transplantation. There are two histological patterns, based on the dimension of fat vacuoles, define steatosis as microvesicular and macrovesicular: while the first is not associated with an increased number of post-OLT complications,[33,34] the secondi s related with a marked increase of PNF and EAD when it involves more than 25-30% of the hepatic mass.[34,35] Again, the mechanism at the basis of the grafts disfunctioning seems to be related with a lower tolerance of the hepatocyte to cold and warm ischemia.[18]

The use of DCD donors could represent an effective recruiting pool still not exploited enought. Donation after Cardiac Death (DCD) means that the liver procurement is performed when the dead of the donor is certified following cardiac death criteria. The different categories of DCD donors were established by Maastricht classification[36] that represent an international reference point to verify and compare results from transplantation activity in different clinical conditions. That classification was further modified in 2013, mantaining the 4 original cathegories, distinguished according to the cause of onset of the cardiac arrest,

introducing other sub-categories (table 4).[37] Donors in the categories 1 and 2 are defined as "uncontrolled" because they include cases of unexpected and sudden cardiac arrest, with an ineffective attempt of resuscitation; thus planning of procurement and assessment of donation consent are carried out necessarily after the heartbeat arrest, leading inevitably to a *Warm Ischemia Time* (WIT) that for the liver is so long that the transplantation of the organ is impossible.

I	Not testified cardiovascular arrest	IA	In-hospital	Uncontrolled
		IB	Out-of-hospital	
II	Testified cardiovascular arrest	IIA	In-hospital	Uncontrolled
		IIB	Out-of -hospital	
III	Expected cardiovascular death			Controlled
IV	Alternative diagnosis of death in course of procedure	IVA	(a)Cardiovascular death during or after definition of death	Uncontrolled
			(b)Cardiovascular death after definition of death with planned cardiac arrest following life support withdrawal	Controlled
		IVB	Death in course of ECLS (ECMO *prior to death*)	Partially controlled

Table 4. Categories of cardiac death donors according to Maastricht, and sub-categories according to "DCD International Workshop, Paris, 2013".

Instead, category 3 donors are called "controlled" because the death happens in an expected way, as a consequence of the planned withdrawal of life support; in this case it is possible to activate the procurement team in advance, keeping the WIT to a minimum. At the same time the WIT, regardless of the way of death, strictly depends on the time needed to certify the death: in Italy 20 minutes electrocardiographic track is needed (while in the majority of european countries this time is limited to 5-10 minutes), consequently the obliged prolonged exposition to the warm ischemia is detrimental for DCD organs, limiting their use for transplantation in the majority of cases.

A WIT lower than 20-30 minutes does not excessively affect graft functioning and guarantees adequate results after transplantation, on the other side the exceeding of this time limit implies an increased number of complications, with a PNF rate around 50% if ischemia time is 30 minutes, until 100% if ischemia is 60 minutes.[38,39]

EAD also is dramatically increased, with negative impact on graft survival and mortality of the patient.[40] Moreover it was proven that the duration of WIT is directly related with the rate of biliary post-OLT complications, that occurr in about 30% of cases in transplantation with DCD livers.[41–43] However, on one side the use of these organs leads to an increased risk procedure related, on the other side the transplantation of DCD livers represents the most effective possibility to expand donor pool, as demostrated by the increased number of transplantations in that countries where procurements of DCD organs have become ordinarily performed in clinical practice. For example in UK 39% of deceased donors are DCD, and these organs are used in about 30-40% of OLT.[44] In conclusion, in the context of a great potential DCD organs usage for shortening of waiting list time, it is clear the necessity of developing new methods for the purpose of a better preservation or improvement of the quality of marginal livers, in order to guarantee a greater safety in transplantation procedure and a reduction of complications.

3. ISCHEMIA-REPERFUSION INJURY

3.1 Physiopathology of Ischemia Reperfusion Injury

Ischemia-reperfusion Injury (IRI) is a series of pathological processes that occurs as a result of reduction of blood supply to an organ or a tissue for a while, followed by the restoration of blood and oxygen delivery.[45]

The extent of IRI damage is directly proportional to the extension of the involved tissue and, particularly, to the duration of ischemic phase. Furthermore cellular distress is strictly dependent to the temperature at which IRI takes place. For this reason it is usual to distinguish cold ischemia from warm ischemia. The second, indeed, takes place at 37°C and is associated with a strong tissues suffering dued to the loss of normal metabolic activity of the cell; instead the first occurs below 10°C, this condition allows to mitigate IRI because metabolic processes and cellular need of ATP are significantly reduced at low temepratures. For this reason hypothermia is commonly used to prevent detrimental effects of warm ischemia in many settings, such as management of trauma and transplantation surgery.[46,47]

From a pathophysiologic point of view, the distinction is made between two different phases of hepatic injury caused by the ischemic damage. The first represents the period of actual ischemia and it is characterized by the metabolic switch to an anaerobic cellular respiration. This process leads to an increased production of lactate and consequently a gradual pH decline. The accumulation of hydrogen ions (H^+) implies the activation of Na+/H+ exchanger and consequently an intracellular accumulation of Na^+. Sodium attracts intercellular fluids through osmosis, causing cell swelling and endoplasmic reticulum dilation. At the same time in the space of 3-4 minutes ATP syntesis dramatically decreases as a result of oxidative phosphorylation block caused by oxygen deficit. ATP depletion inactivates ATP dependent mechanisms, including Na^+/K^+ ATPasi and Ca^{2+} ATPasi pumps, with a consequent accumulation of ions. The increase of Ca^{2+} concentration is even more favored by the decreased reuptake of endoplasmic reticulum.[48]

The second phase, called "reperfusion", occur as a consequence of the re-oxygenation of the organ and it is stricly dependent to the molecular alterations developed by the tissue during the ischemia time.

Underlying mechanisms of reperfusion injury are multifactorial, complex and they include Calcium accumulation, reactive oxygen species (ROS) formation, mitochondrial and microvascular dysfunction and the inflammatory response. Reperfusion phase consists of two different responses to IRI: an early response and a late response.[48]

Early reperfusion phase

The main mechanisms of this phase are the activation of Kupffer's cells, that are macrophages specific of liver located in the sinusoidal space, and the consequent production of ROS (figure 7), responsables of oxydative injury.

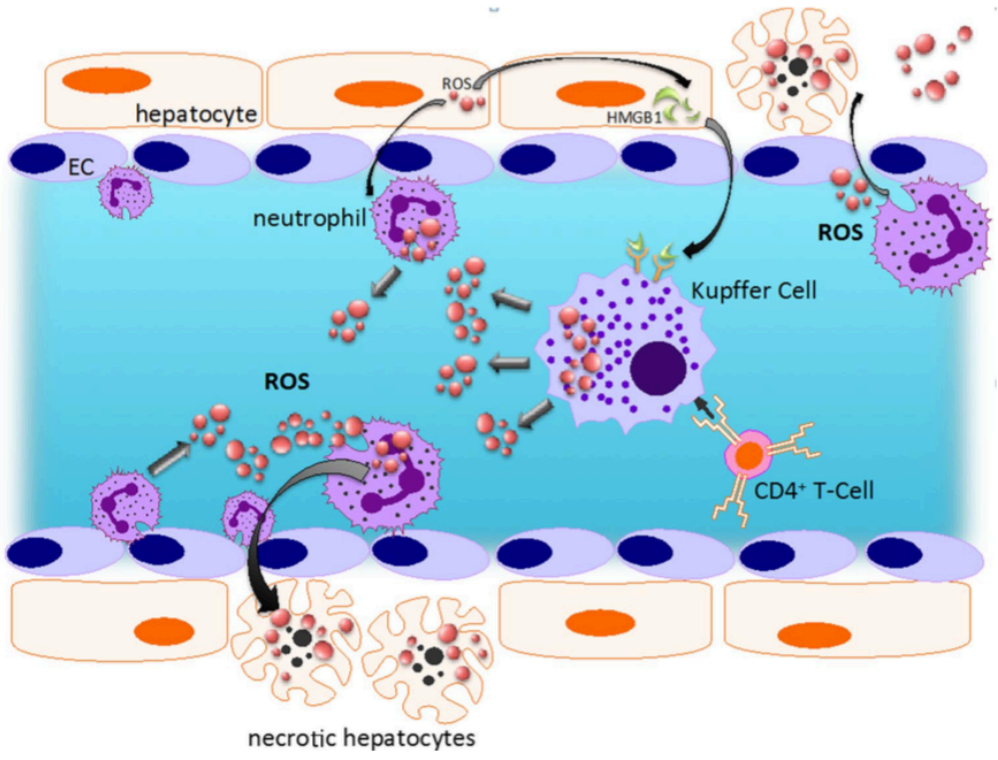


Figure 7. Kupfer's cells are the main site of ROS production during the early phase of reperfusion injury. Image adjusted from "*Molecular responses to ischemia and reperfusion in the liver*. Quesnelle KM et al., 2015".[45]

Kupffer cells are activated by different mechanisms involving molecules and pathways typical of the innate immune response (figure 8). The main factors involved are listed below:

- Complement: it includes a set of circulating small proteins with a central role for inflamation and immune defence. During IRI, complement cascade is stimulated by cellular proteins released immediately after the reperfusion and it is responsible for a direct damage through Kupffer's cell activation.
- Toll-Like Receptors (TLR): these receptors are key mediators as trigger of innate immune response to IRI. TLRs are placed on the surface of many type of hepatic cells, such as Kupffer's cells, dendritic cells and hepatocytes. They are able to recognize the typical molecular profile of injuried cells, called "Damage Associated Molecular Patterns" (DAMP). During IRI, the hepatocellular oxidative stress mediates the release of High Mobility Group Box 1 (HMGB1), one of the most known DAMP. The level of HMGB1 increases already in one hour from reperfusion. These factors act as ligands for TLR, which in turn trigger a signal cascade that activate the trascription of pro-inflamation mediators, including TNF-a, IL-6, ICAM-1, INF1 and CXCL10.
- NF-kB: it is an important transcription factor for the expression of genes involved in inflamation. It is composed by proteins of Rel family, that share an homologous amino acid sequence called "Rel homology domain". This is necessary for dimerisation, DNA binding and protein IkB binding (NF-kB inibitor). During the early phase, NF-kB is activated by the oxidative stress and by inflammatory stimulation and its role in the response to IRI depends on the type of cell: in Kupfer's cells NF-kB activation promotes the expression of TNF-a and IL-6; in hepatocytes NF-kB activation guides the production of TNF-a; finally the activation of NF-kB in endotelial cells causes the expression of chemokine of the family IL-8 and of adhesion molecules as E-selectina, ICAM-1 e VCAM-1.
- Inflammatory cytokine: citokine cascade during IRI starts with the up-regulation of IL-12 e IL-23. The font of IL-12 and IL-23 has not been identified yet, but kupffer's cells and hepatic stellate cells are the main candidate for the production of these molecules[49]. IL-12 e IL-23 amplify the inflammatory response by

stimulating the expression of INF-γ e TNF-α. The latter has been recognized for a long time as the most important mediator of hepatic inflammatory response. TNF-α is transcribed by many cells in the liver, but its release by kupfer's cells is massive and can be detected just after reperfusion. This cytokine stimulates hepatocytes and Kupffer's cells to produce chemotactic molecules for neutrophils, expecially chemokines from the family CXC. Furthermore TNF-α stimulates the trascription of adhesion molecules ICAM-1, VCAM-1 and P-selectine on the surface of endothelial vascular cells. The importance of TNF-α during IRI is proven by the fact that its inhibition is able to stop almost completely the inflammatory response and the hepatic damage following IRI.[50,51]

In addition to the T-independent mechanisms shown before, also the adaptive immunitary response takes part in IRI, albeit it acts to a lesser extent compared to the innate response. CD4+ T Lymphocytes are one of the first group of cells to be recruited in the liver after reperfusion and they partecipate to the spread of injury releasing IL-17; this cytokine promote chemokines release by other types of cells, included Kupfer's cells, epithelial cells, fibroblasts and endothelial cells and leads to neutrophils recriutment (figure 8).

Finally, during the early phase of reperfusion response, activated Kupfer's cells are the main source of ROS, the accumulation of which represents the central element in IRI pathogenesys: indeed the reperfusion of ischemic tissues involves the production of a large amount of ROS, that, for their hightly reactive action, can oxidate any molecule inside the cell. Oxidative damage is a common mechanism, that can be observed during every ischemic or hypoxic injuries, that carries to alterations in oxidation/reduction ohomeostasis. In particular, during reperfusion, oxygen is rapidly reintroduced inside the cells, that have just swithched to an anaerobic respiration. As a result of that switch the oxidative phosphorylation is interrupted with a consequent accumulation of carrier molecules of reduced electrons. When oxygen concentration becomes suddenly normal during reperfusion, carrier molecules give way rapidly their electrons to the oxygen determining the peak of ROS.

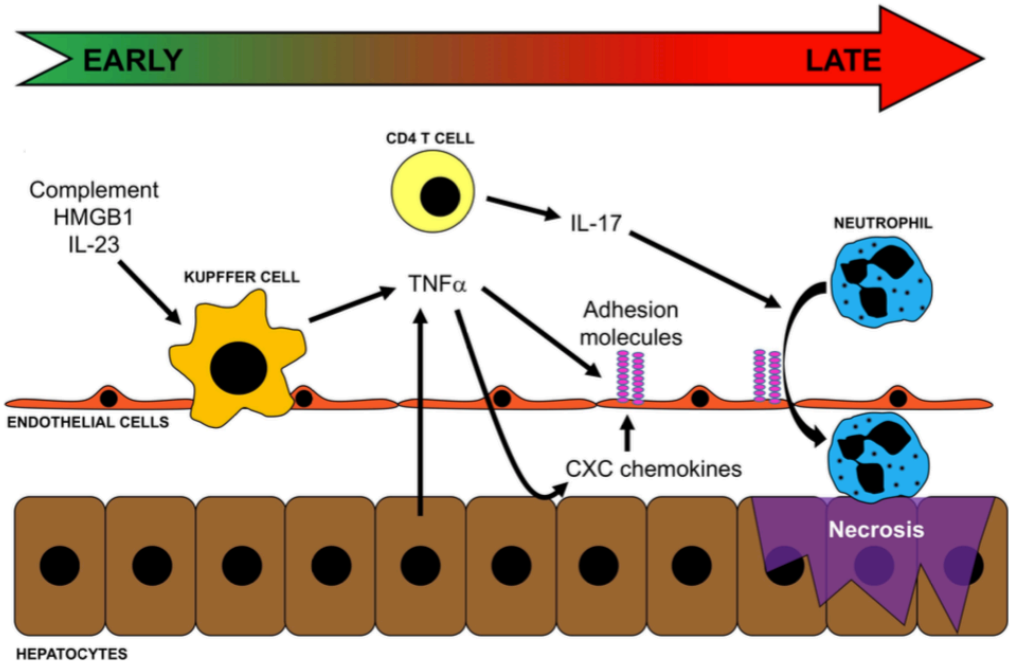


Figure 8. Early and late phase of reperfusion injury whith molecular mechanisms involved. Imagine adapted by "*Hepatic Ischemia/Reperfusion: Mechanisms of Tissue Injury, Repair, and Regeneration,* Konishi T et al., 2017".[48]

ROS synthesis takes place mainly inside mitochondria with the production of superoxide ion (O_2^-), that is immediately converted in hydrogen peroxide (H_2O_2) through mitochondrial superoxide-dismutase with the following reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Hydrogen peroxide, that is not a radical, has a mild oxidant power and is able to pass through cellular membranes reaching cytoplasmic compartment.

H_2O_2 in pathologic conditions and in the presence of iron ions, is trasformed in the hydroxy radical (·OH), a free radical. Another possible reaction is the Haber-Weiss reaction, this too is amplified in pathologic conditions: $H_2O_2 + O_2^- \rightarrow \cdot OH + OH^+ + O_2$. Finally, also nitric oxide (NO) can react a) with O_2 forming nitrous groups, b) with sulphydryl groups or c) with superoxide anion forming peroxynitrite, that is one of the most powerful biological oxidant known. Oxidative stress caused by ROS is expressed by the oxidation, the creation of intra or intermolecular S-S bridges, or by protein cross-linking with the subsequent genesis of neo-antigens. In particular the injury is visible on three levels:

a) Damage of cellular macromolecules (lipid membranes, proteins and DNA);
b) Reduction of NO bioavailability because of the reaction with O_2^-;

c) Direct and indirect effect on intracellular signalling mechanisms (altering redox systems).

Despite mitochondria is the main place of ROS production inside the cell, they are also formed outside mitochondria by a group of enzymatic units placed on the plasma membrane. These are NADPH-oxidase, 5-lipoxygenase and xanthine-ossidasi.

Late reperfusion phase

The delayed or late phase is characterized by the process of neutrophils recruitment and the cellular death as a consequence of inflamation. Recruiting mechanisms depend on the circulating neutrophils' ability to stick in the vascular endothelium and cross the wall of blood vessels. This process is mediated by chemotactic agents and adhesion molecules, both produced during the early phase.

Once neutrophils reach the target tissue, they trigger a massive production of ROS: the interaction between an hepatocyte and a neutrophil leads to the activation of NADPH oxidase. The oxidation of NADPH releases an electron that reduces molecular oxygen forming superoxide anion, that is substrate for the production of radicals. ROS produced by neutrophils spread inside hepatocytes and cause mitochondrial dysfunction followed by Calcium accumulation and mitochondrial permeability transition pore (mPTP) opening. It leads to the so called mitochondrial permeability transition (MPT), from which inevitably the cellular death comes.

In addition to ROS generation, activated neutrophils realease many other mediators through a granular exocytosis mechanism. Neutrophils degranulation means the release of considerable quantities of proteases (such as elastase, G cathepsin, heparinase, collagenase) and hydrolytic enzymes that can directly operate with a cytotoxic action on hepatocytes.

Cellular death consequent to IRI

The way of death of hepatocytes consequent to IRI is still under study. In particular two modalities of cellular death following an ischemic damage have been described so far: apoptosis and necrosis. Distinctive signs of apoptosis are cellular shrinkage, chromatin condensation, nuclear fragmentation and apoptotic bodies formation.

Instead necrosis is characterized by mitochondrial and cellular swelling and, as a consequence, by the loss of structural integrity of plasma membrane, that ends with vacuolization.[45]

Hepatocellular necrosis observed during IRI is mainly supported by MPT, a process triggered by ROS, which leads to pores formation on the mitochondrial membrane. This causes ions entrance and, consequently, mitochondria uncoupling, ATP depletion and the loss of mitochondrial membrane potential. Also the impaired homeostasis of Calcium promotes MTP both direcly and indirectly through the activation of PCK.[52]

On the other hand MTP activation results in a rapid trigger of apoptotic cascades. The increased mitochondrial permeability causes the release of many pro-apoptotic proteins inside cytoplasm, by inducing the release of AIF (Apoptosis Inducing Factor) and the production of cytochrome C, a member of apoptosome.[53]

3.2 Impact of IRI on DCD livers

Livers from DCD donors are the most sensitive organs to IRI. Longer is the duration of ischemic phase, greater will be tissue injury following reperfusion,[54] because the accumulation of electrons and ATP depletion during prolonged anoxia lead to a greater inflamatory response and an higher ROS production, compared to not marginal organs.[48] This process results in an increased cell necrosis and apoptosis, until an irreversible hepatocyte degeneration if the warm ischemia duration exceeds 45 minutes.[54,55]

Molecular mechanisms are reflected in organ functionality after transplantation. In a cohort study conducted on more than 200 OLT performed with DCD grafts, EAD rate was 39,5%, with consequent negative results in term of graft and patients survival.[40] An european analysis of long term follow-up (1, 5 and 10 years) on patients transplanted with DCD livers confirmed this trend, demostrating an increased number of complications and organ failure when WIT is over 25 minutes.[39] Finally, a meta-analysis on 25 studies reported a considerable increase of biliary complications and ischemic cholangiopaties in DCD organs, together with an higher mortality 3 year after transplantation.[56]

4. GRAFT PRESERVATION

4.1 Static Cold Storage

Preservation phase is that period of transplantation process that elapses between the organ procurement and the transplantation in the recipient. During this time, that includes the transport of the graft from the donor's hospital to the transplantation center, all the strategies that allow the maximum limitation of ischemic injuries are put in place. The organ cooling applied through the Static Cold Storage (SCS) represents still today the gold standard for preservation. It takes advantages of the reduction of enzymatic activity that normally happens at temperatures near 0°C; rapid cooling of the graft, together with the protective effect of specific solutions, slow down the hepatocellular metabolism, reducing ATP depletion and the loss of cellular integrity.[46] Practically SCS consists in the washing and the immersion of the graft into a preservation fluid and its preservation with ice at about 4°C; at this temerature the cellular activity is reduced by 90%.[47] During the years many different preservation solutions were developed, they are different for viscosity, ionic composition and costs. The most used are the "University of Wisconsin Solution", the "Celsior Solution", the "Histidine-Tryptophan-Ketoglutarate Solution", and the "Institute Georges Lopez-1 Solution".[57]

On the other hand low temperatures damage hepatic microcirculation and cholangiocytes, and cause toxic metabolites release that trigger post-reperfusion injury (cfr. chapter 3).[58,59] Hence the duration of cold preservation is direcly related with PNF incidence[60] and the transport and the transplantation should be performed as quickly as possible. Furthermore recent studies have pointed out that with the advent of marginal organs the SCS does not represent anymore the ideal preservation method. During cold ischemia time, indeed, the cellular activity is reduced but not totally inhibited and a minimal anaerobic metabolism persists and it lead to ATP depletion and deregulation of ionic homeostasis.[61] This injury is reversible and well tolerated by good quality organs, but not by DCD organs, that seem to be more prone to develop oxidative stress and micro-circulation alterations, with consequent negative effects in terms of complications and post-OLT mortality.[38,40,62–64]

4.2 Machine perfusion for liver preservation

A valid alternative to SCS preservation is the use of extracorporeal Machine Perfusion (MP).

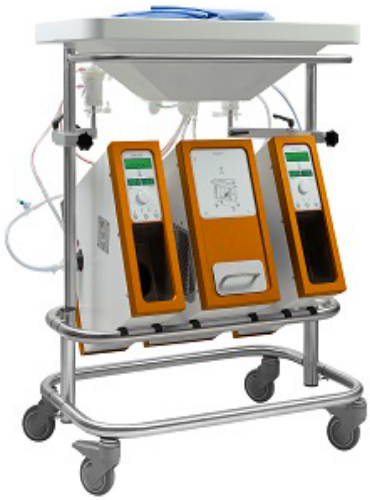
In 1930 Alexis Carrel published a paper about thyroid normotermic perfusion and for the first time introduced the ambitious and fascinating concept of ex vivo preservation of whole organs.[65] In 60's-70's the first machines were patented: in 1963 the first hypothermic perfusion experiment were carried on cadavers, in order to temporary preserve organs for transplantation.[66] In the following years, in an effort to maximize the survival of organs with MP, groups guided by MacLean and Belzer demostrating the possibility to carry on an organ perfusion on dogs models for 24 hours.[67,68] In 1968, using hypothermic MP with omologous blood as a perfusate and hyperbaric oxygen, the first OLT were successfully performed on dogs after a perfusion of 8 hours.[69] Five years later the limit of 24 hours was reached. [70] In 1980 Kamada proposed the use of an emulsion containing fluorocarbon as an oxygen carrier to improve energetic support of the graft during MP.[71] The encouraging results of these studies prove the unquestionable advantage of a continuous oxygen and nutrients supply during the preservation phase. Furthermore the great interest about MP at that time was encouraged by the fact that an effective technique to guarantee organ preservation longer than 8 hours did had not been discovered iet. Thanks to the invention of Belzer's solution,[5] the benefits introduced, including ease of application and lower costs, caused an arrest in studying expensive and bulky machine perfusion.[72]

Recently the increased necessity of livers for transplantation and the possibility to use DCD organs for donors pool expansion have raised new interests for MP field. Technological development of XXI century allowed the construction of smaller and easier machines, that provide oxigen and nutrients administration during the perfusion, extending the preservation time. This gives a logistic advantage, expanding the time for organ transport, but also a technical benefit, because of the possibility to real time monitoring graft's quality that allows an objective evaluation of transplantation feasibility. Moreover the fact that SCS is not very effective for ECD organs conservation makes the perfusion with MP the main candidate for maginal organs reconditioning and preservation for their use in transplantation.

Indeed the superiority of MP over SCS in marginal organs protection has already been demostrated in many sudies.[73–80]

There are many differences between MP models, but in general the basal components of an extracorporeal preservation circuit are a perfusion chamber, in which the organ is placed and connected to the circuit, an oxygenator, one or mor pumps that move the preservation solution following flow or pressure parameters setted by the operator, and a thermoregulator. The characteristics that influence mostly the perfusion protocol are: the device portability and the temperature of perfusion. The possibility to move the MP to the donor's hospital allows to decrease ischemia time but makes the whole procedure technically and logistically more difficult. On the other hand using untrasportable MP precludes the possibility to avoid SCS but makes the connection procedure easier because it is performed in the transplantation center. For what concerning the termic regulation of perfusion, nowadays the two most common techniques differ mainly according to the temperature at which the graft is maintained during perfusion.

The MP currently on the market and available to be used in clinical practice for liver grafts are the following:



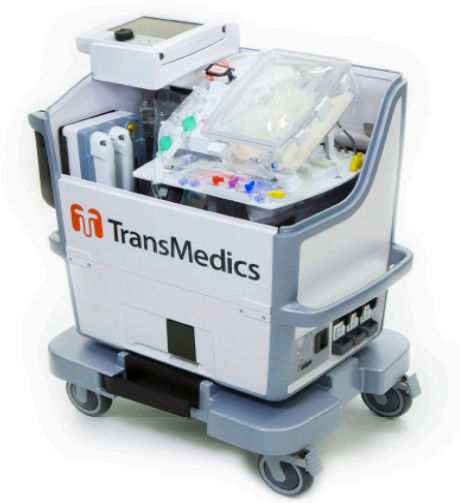
ORGAN ASSIST LIVERASSIST

Liver Assist Œ (OrganAssist) it provides a pulsatile flow to the hepatic artery (HA) and a continuous flow to the portal vein (PV). For this reason it is equipped with two different circuits with different pumps and hollow fiber oxigenators. The temperature settings are not fixed, and they can be manually regulated within a range between 10°C and 38°C. The vena cava is left open and free to drain in the perfusion chamber. The device is not portable, and it can be used only in the transplantation center. It is patented for perfusion of 6 hours.

OrganOx© (Metra) is a portable normotermic perfusion device. It provides a continuous flow to HA and PV, using a single pump that carries the perfusate directly to the artery and through a reservoir to the PV. The system is almost completely automatic, the blood gas analysis is integrated and the regulation of arterial pressure and oxygen supply is controlled. Temperature is settled automatically at 37°C. The system is designed to a length of perfusion until 24 hours and it can be transported to the procurement hospital.

LifePort Liver Transporter® (Organ Recovery Systems) design is very similar to the machine perfusion used for kidney transport that is diffusely utilized nowadays. It allows exclusively hypothermic perfusion and is not equipped with active oxygenation system. It is designed to be easily transported to the donor's center. It's clinical application is currently limited.

Organ Care System™: the system from *TransMedics Organ Care* includes portable normothermic perfusion devices designed for heart, lung and liver. The use of this machine is widely consolidated both in heart and in lung transplantation nowadays. On the contrary the clinical feasibility studies for liver perfusion are still in progress.



TransMedics®
Bringing new life to organ transplant™

4.3 *Hypothermic Machine Perfusion*

Hypothermic Machines Perfusion (HMP) are preservation circuits that maintain the organ at low temperatures, between 0°C and 12°C. HMP take advantage from the reduction of cellular metabolic activity in hypothermic conditions and they were the first machines used for organ preservation.[68]

Studies on large animals have demostrated the possibility to use the University of Wisconsin Solution (UWS) to preserve ex vivo the organ for 72h, with a pulsatile flow and a portal pressure between 16-18 mmHg.[81] Recently Guarrera's group proposed the use of Vasosol solution, that consists in the adding of aminoacids and prostaglandin E1 to UWS in order to guarantee an energetic and antioxidant support to the liver.[82] Again using Vasosol, the same group published the first clinical trial demostrating that HMP is a safe and effective preservation method that compared to SCS is able to reduce serum AST, ALT and bilirubine in trasplanted patients, to decrease biliary complication and to lower hospitalization time.[73] In a following analysis HMP was able to reduce the expression of inflammatory cytokines, chemokines, adhesion molecules, oxidative stress markers and acute phase proteins compared to livers preserved by SCS.[74] However none of the two studies

have demostated significative differences about the most clinically relevant endpoints, such as PNF, EAD, and post-OLT survival.

In 2006, Philipp Dutkowski proposed an additional variation in HMP inventing the Hypothermic Oxygenated Perfusion (HOPE), a technique that provides the active oxigenation of the perfusate reaching very hight values of pO_2 (300-600 mmHg). Compared to not oxigenated HMP, HOPE is able to prevent post-perfusion mitochondrial distress and then to reduce IRI thanks to a lower activation of Kupffer's cells.[83] That mechanism seems to be even more effective in the preservation of sub-optimal organs. In 2014, indeed, based on a great number of preclinical experimental studies,[84–86] Dutkowski's team performed the first human study with DCD livers treated with HOPE.[87] In the presented series, DCD livers treated with HOPE resulted in an excellent post-OLT functionality: liver enzymes, renal functioning, lenght of stay in ICU were comparable or even better than the matched controls (brain death donors). After a median follow-up of 8-9 month none of the patients developed biliary complications. The same group continued clinical studies with HOPE demostrating that this technique is more effective in preserving DCD livers compared with SCS.[75] Indeed HOPE allowed to reduce post-operative AST peak, ALP, bilirubine and INR levels. The same study also observed in livers treated with HOPE a lower incidence of biliary complications, confirming the results of the previous series.[87] In Italy a phase I trial was carried on with HOPE and it demostrated a lower AST peak during the first week post-OLT and a significative decrease of EAD incidence in the perfused livers.[88]

Simultaneously, at Groeningen University, Robert Porte carried on a series of studies using HOPE in a model of perfusion of both HA and PV (Dual Hypothermic Oxygenated Perfusion - DHOPE). Since the biliary tree is vascularized mainly by the arterial system,[89] the rationale of DHOPE technique is the effort to reduce to the minimum biliary complications, that are nowadays the most critical aspect of liver transplantation with DCDs.[90,91] On the other hand the presence of numerous collateral vessels connecting arterial circle with portal circle seems to make the perfusion only throught portal vein effective enough [92]. For this reason the effective advantage provided by DHOPE is still debated, considering also the highter complexity of the procedure dued to arterial cannulation.[92,93] Groeningen group has

recently published results of a case-control study to estabilish the clinical feasibility of DHOPE with DCD livers:[76] the machine led to a better response to IRI documented by a lower depletion of ATP and a better histological aspect of the biliary epithelium in livers treated with DHOPE.[76,94] On the contrary in the 20 organs preserved with SCS a re-transplantion was needed in 25% of cases.[76] This preliminar study, although with limited statistical significance for major outcomes, lays the basis for further clinical investigations.[95]

Nowadays in Europe three randomized controlled clinical studies are ongoing, their characteristics are resumed in table 5. Results of these trials will enable a dedefinitive evaluation of the benefits of hypothermic perfusion for DCD organs preserving. Anyway from preliminary results it is clear that HMP, HOPE and DHOPE are relatively easy precedures to perform and effective, expecially when they are applied at the end of SCS period, with the aim of graft quality improvement and reducing of ischemic injury. On the other hand one of the main limit of cold MP is the impossibility to evaluate the quality of the graft during perfusion. Today the only limited evidences of predittivity of graft viability regards hemodynamic parameters such as flow and resistance measured during HMP.[96]

Also in the Transplantation Center of Turin a protocol involving HOPE for ECD livers reconditioning is currently in progress.[97]

Study	**Zurig NCT01317342 April 2015**	**Groningen NCT02584283 October 2015**	**Aachen NCT03124641 Dicember 2017**
Comparison	HOPE vs SCS	DHOPE vs SCS	HOPE vs SCS
Number	85 vs 85	78 vs 78	23 vs 23
Donation type	DBD+ECD	DCD	DBD+ECD
Device	LiverAssist, OrganAssist Œ	LiverAssist, OrganAssist Œ	LiverAssist, OrganAssist Œ
Temperature	4-6°C	10°C	10°C
Perfusion type	HOPE	DHOPE (2h)	HOPE (1-2h)
Perfusate	IGL-1 Solution	Belzer Solution supplemented with glutathione	Belzer Solution
Endpoints	Major postoperative complications	NAS (*nonanastomotic biliary strictures*)	Early graft injury (peak of ALT)

	(Clavien>3) and CCI (*comprehensive complication index*)		

Table 5. Randomized clinical trials currently ongoing for clinical validation of HOPE.

4.4 Normothermic Machine Perfusion

Normothermic Machine Perfusion (NMP) are devices inside which the liver can be connected ex vivo to a closed circuit and preserved at physiologic temperature by continuous administration of oxygen and nutrients. Initially NMP circuits were built using cardiopulmonary by-pass components. The main components are a blood reservoir, one or two pumps, an oxygenator and an heat exchanger. Perfusate is usually composed by solutions containing packed red blood cells in order to provide an effective oxigen delivery. A costant blood gas analysis provides pO2 and pCO2 levels control, making easier the preservation of acid-base equilibrium. During NMP the metabolic activity of the liver is preserved and, therefore, a set of pumps continuously infuses glucose, aminoacids, heparin, insuline and bile salts in order to guarantee the physiological hepatic synthesis and avoid clots.

Based on the characteristics described before, it is evident that the major NMP advantages are:

a) Prolongation of perfusion time

Ideally NMP is able to preserve an organ ex vivo until 24 hours; resulting in the possibility to approciate the transplantation procedure in a more controlled and organized way, with a better utilization of operatory theater, of healthcare professional, and improving of the list management. The extension of preservation time allows also to have an useful time for applying therapeutic reconditioning protocols during NMP.

b) Evaluation of graft viability

The ability to predict the post-transplantation outcome is an attractive perspective that distinguishes the NMP by the SCS and the HMP.

For example in UK, in the last 10 years, the number of liver procured but not transplanted is doubled.[44] Organs are usually discarded esclusively as a result of a macroscopic evaluation taking into account also donor's characteristics; in SCS preservation the presence of objective predictors of post-OLT organ's funtionality is lacking. On the contrary NMP provides the opportunity to really evaluate liver's functionality during the preservation and it could potentially increase the predictive capability of post transplantation results. Some surrogate markers of viability proposed, in addition to macroscopic aspect of the graft, are: lactate clearance, haemodynamic stability of flow and pressure, the capacity of mantaining acid-base homeostasis, transaminase levels in the perfusate, the quantity of bile produced and bile pH.

c) Liver reconditioning before transplantation

Furthermore NMP provides the possibility to treat the graft ex vivo during the preservation phase. This prerogative is exclusive for the NMP, because it is capable to maintain the organ metabolically active, and then sensitive to the administration of pharmacological therapies aimed at improving the graft functionality. This approach is called "organ reconditioning" and represents a new possibility for reducing the number of discharged organs.

The first studies of normothermic liver perfusion date from the end of 1800. Circuits designed at that time were aimed to recreate experimental situations in order to study liver physiology and physiopathology;[98] it's only in '80s that the first animal models of NMP were applied in the transplantation setting.[99] In the first 2000 are reported the first NMP devices capable to preserve organs for many hours with post-transplantation satisfying results. Many studies defined technical characteristics needed for experimental use of NMP in rats and pigs.[100–102] Based on these works, the idea of the application of NMP on marginal organs rised in clinical setting, and the first experiments started in order to evaluate the effectiveness of NMP in donation at risk, in order to expand donor pool.

Study	Species	Number of CS (duration in h)	Number of NMP (duration in h)	Outcomes
Tolboom 2008[103]	Rat	6 (6)	6 (6)	AST, ALT, urea and bilirubine levels; bile production; oxygen consumption; post-OLT survival.
Tolboom 2009[104]	Rat	6 (5)	6 (6)	AST, ALT, urea, bilirubine, glucose and total proteins levels; bile production; oxygen consumption; histological aspect; post-OLT survival.
op den Dries 2016 [105]	Rat	7-9 (3)	7-9 (3)	AST, ALT and LDH levels; bile production; bile pH, bilirubine level of bile, HCO_3^- bile, γGT bile and LDH bile; portal flow; histological aspect.
Imber 2002[77]	Pig	5 (24)	5 (24)	ALT, ALP, γGT, glucose and factor V; bile production; histological aspect; galattose metabolism; oxygen consumption; emolysis.
Peter 2002[78]	Pig	4 (24)	4 (24)	ALT and glucosie levels; bile production; portal flow.
Nassar 2015[79]	Pig	5 (10)	15 (10)	ALT, AST, LDH and ALP level; bile production; portal flow; histological aspect.
Brockmann 2009[80]	Pig	4 (20)	6 (20)	AST and ALT levels; portal flow; histological aspect; immunohistochemistry; survival.

Table 6. Experimental preclinical studies with NMP

Concerning the small animals, a long series of studies carried on in Harvard by the group of Herman Tolboom and Korkut Uygun have demostrated the superiority of NMP to SCS in DCD liver preserving. In their model of OLT, animals transplanted with organs treated with NMP had a 28-days survival close to 100%, in contrast with a survival of 0% already in the first postoperative day observed in the

group of animals receiving organs preserved in a conventional way (SCS).[103,104,106] NMP resulted also capable to protect the biliary epithelium from the ischemic injury.[105] Experimental studies on pigs, much more similar to the clinical setting beacause they use machines almost equivalent to that used in humans, confirmed what observed in the murine models: in the setting of DCD donation at hight risk (*i.e.* very long warm ischemia time), NMP is the only preservation thecnique able to guarantee the recipient survival post transplantation.[77–80] The main details of the mentioned experimental models are described in table 6.

According with preclinical results, NMP was recently introduced in clinical setting. The first phase 1 study was carried on in UK by the Oxford's group and demonstrated safety and feasibility of NMP in humans.[107] Liver perfused with NMP for a mean time of 9,3 hours, demostrated hemodynamic stability, syntesis functionality, active bile production and pH autoregulation. Compared with SCS, 30-days survival after transplantation was similar, with lower transaminases values (in particular serum AST peak in the first 7 days after OLT). These preliminar results were further confirmed by two subsequent studies when NMP was used to transplant successfully 10 and 9 livers respectively.[108,109] The first RCT that compared the effectiveness of NMP and SCS was completed in Europe.[110] On a group of 220 transplantation analyzed, NMP was able to significantly reduce AST peak in the first post-OLT week, to reduce by half the rate of organ discharged and to increase of 54% preservation time. Despite the promising previous results, there were no improvements in terms of biliary complications, graft survival and patients survival. In order to evidence significant differences in terms of outcomes, additional RCTs with a larger number of patients and a long time follow up are needed (table 7).

Study	**USA (Multicentric) NCT02522871 January 2016**	**USA (Multicentrico) NCT02775162 Giugno 2016**
Comparison	NMP vs SCS	NMP vs SCS
Number	150 vs 150	133 vs 133
Type of donation	DBD+ECD	DBD+ECD
Device	TransMedics OCS™	OrganOx Metra ©

Endpoints	EAD and 30-days complications	EAD

Table 7. RCT today ongoing for the clinical validation of NMP.

4.5 NMP and new reconditioning strategies for marginal grafts

As previously anticipated, NMP is the only perfusion system that offers the possibility to pharmacologically treat the liver during the preservation time (figure 9). The idea of resuscitation or reconditioning for low quality organs is fascinating and, despite it is a very recent and experimental concept, it could represent in the future a possible solution to reduce the gap between the number of donors and patients on the waiting list.

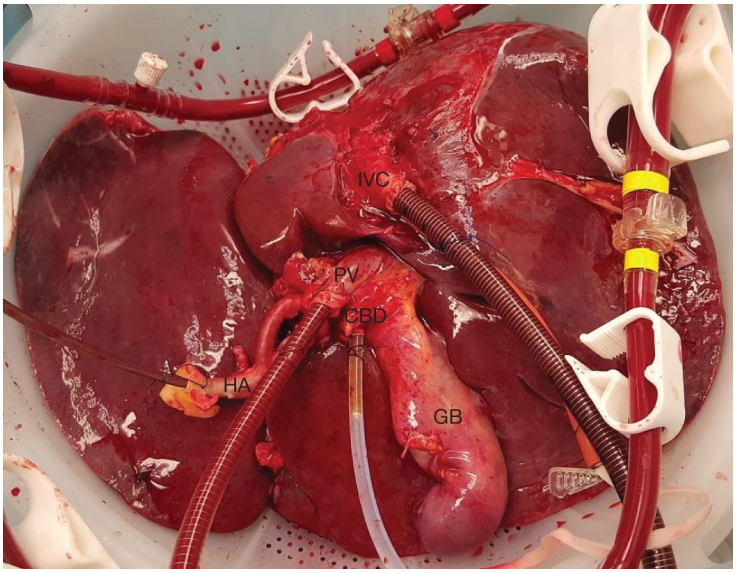


Figure 9: Human liver perfused with NMP. Inferior Vena Cava (IVC); Portal Vein (PV) Hepatic Artery (HA); Common Bile Duct (CBD), Gallbladder (GB). Image adapted from "A randomized trial of normothermic preservation in liver transplantation, Nasralla D et al., 2018".[110]

One of the possible field of application of reconditioning concept is on steatotic livers. Even if the NMP alone leads to an improvement of graft quality,[111,112] some studies were performed with the adding of "defatting" molecules in the perfusate, able to stimulate lipolysis in a short time. Thanks to this thecnique Nagrath et al.[113] demostrated in a murine model the possibility to reduce of 50% the fat content in steatotic livers after only 3 hours of NMP, while in a recent experiment carried on discharged human livers was described in one of the two livers treated a reduction of

steatosis of 10%.[114] However the evidences in the application of this procedure are still extremely limited and their effectiveness is debated.[115]

In 2017 a study on pigs was published about the possibility of inhibition of viral replication in livers infected by HCV using NMP circuit. It allowed to convey antisense miRNA (miravirsen) that is able to inactivate miRNA122, a viral molecule necessary for replication.[116] It is the first proof of effectiveness of NMP used to treat the liver with a genic therapy during reperfusion phase.

Concerning reconditioning in the experimental setting of DCD livers, today only three papers were published, these demostrated the possibility of treating ischemic graft with stem cell derived products respectively for lungs, kidney and liver. Stone et al. used NMP to recondition DCD lungs from rats with Mesenchymal Stromal Cell-derived Extracellular Vesicles (MSC-EV), and observed, in treated organs, better hemodynamic parameters ad a reduction of pulmunary neutrophil infiltrate and edema.[117]

Using HMP, University of Pavia's group demostrated the reduction of kidney ischemic injury by administration of MSC-EV. DCD kidneys perfused with MSC-EV showed significantly lower levels of lactate, LDH, malondialdeide and glucose in the perfusate, a better histologic aspect and an up-regulation of genes involved in cellular metabolism and in ischemia response.[118]

About liver, our group has recently proved the possibility to use a stem cells derived therapy also for liver reconditioning, by means of the administration of extracellular vescicles derived from hepatic stem cells (cfr. chapter 5). In a murine model of hypoxic NMP. We observed that the vescicles were able to integrate in liver parenchyma within 4 hours of perfusion, providing a protective action for hepatocytes. In treated livers we found a significant reduction of AST and LDH in the perfusate and a reduction of necrosis and apoptosis into the tissue specimens.[119] These are very promising results, although very limited; further studies are needed to confirm what we observed and to implement these experimental models with procedures as similar as possible to the clinical reality.[120]

5. HUMAN LIVER STEM CELLS AND THEIR DERIVATES

5.1 Stem cells

A cell is defined "stem cell" when it has replicative immortality and self-renewal ability. They are, indeed, not terminally differentiated, able to divide unlimitedly during the time and to generate at every cycle two daughter cells with different destiny (asymmetric division): one of the two remains identical to the mother cell and guarantees the renewal of the staminal pool of the tissue, the second, usually owning a strong replicative potential, proceeds to the following steps of differentiation (figure 10).[121]

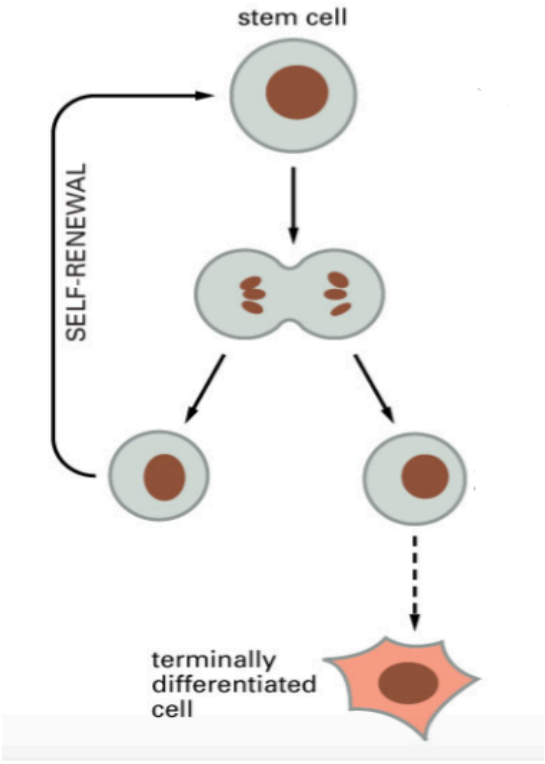


Figure 10. The asymmetric division of stem cells. Image adapted by "*Molecular Biology of the Cell*, Alberts B, VI edition".[121]

Stem cells are divided according to their potency, that means the ability to origin different lines of differentiated cells:[122]

- Totipotent stem cells: these cells are able to generate a whole mature organism, because they are capable to differentiate in any different type of embryonal or extraembryonal cell. For example, the zygote and the embryonal cells within the first two/three cellular division (4-8 cells embryon) are totipotent cells.
- Pluripotent stem cells: they can differentiate in one of the three germinal layers and consequently they can originate any type of cell in the adult being but not the extraembryonal tissues. For example the embryonal cells at the stadium of

blastocyst and the induced pluripotent stem cells (iPS) are in this cathegory. iPS are the result of a revolutionary finding by the group of Shinya Yamanaka, that could produce stem cells with totipotent characteristics by the inclusion of 4 genes codifying growth factors (Oct4, Sox2, Klf4, c-myc) in adult fibroblasts.[123]

- Multipotent stem cells: these are cells able to differentiate in a limited number of cellular types within the same differentiation line. Adipose tissue stem cell or haematopoietic stem cells are examples of multipotent stem cells. The latter can originate the different cells composing blood, but not other cellular types.
- Oligopotent stem cells: they can differentiate in only few cellular types. Under this category there are lymphoid and myeloid stem cells.
- Unipotent stem cells: from these only one type of cell can oryginate, the same type that constitutes the tissue they belong to. They are different from not stem cells for their capacity of self-renewal. An example of unipotent stem cell is in the muscular tissue.

Adult stem cells

Adult Stem Cells (ASC) are a type of stem cells that can be isolated from tissues of organisms that have completed their embryonal developement. They are also known as somatic or tissue-specific stem cells. Their role is to guarantee the renewal of their tissue of belonging in physiologic conditions or as a response to an injury. Usually these are cells with multipotent, oligopotent or unipotent characteristics; the pluripotent adult stem cells are infact very rare.

Between the most known examples of ASC there are the Mesenchimal Stem Cells (MSC), haematopoietic stem cells, adipose tissue derived stem cells and endothelial stem cells. However other groups of ASC were identified also in other tissues and organs, such as brain, scheletric muscle, skin, spleen, pancreas and liver, proving the fact that these cells have a key role in tissues regeneration and homeostasis.[124]

It is commonly believed that ASC are limited in their differentiation capability following their tissue of origin, however many studies suggested that they are provided with a sort of plasticity and can differentiate into cells belonging to different lines.[125] Thanks to these qualities, together with the fact that their

production does not require embryons, ASC are considered among the most interesting options in the field of regenerative medicine.[126]

5.2 *Human Liver Stem Cells*

The liver is an organ characterized by unique regenerative capability, probably already known in ancient times (Prometheus' Myth), and described for the first time in 1931 by Higgins and Anderson, that observed the complete restoration of the whole hepatic mass after subtotal hepatectomy in the rat.[127]

Mechanisms underlying the embryonal and fetal developement of the liver have been widely described, and involve hepatoblasts as key players, that are progenitors cells able to differentiate in hepatocytes and cholangicytes by means of a paracrine cell-to-cell interaction.[128]

On the contrary in adult liver molecular basis of regeneration are still under study and debated. It is important, first of all, to distinguish between the physiologic turnover of hepatocytes and the reaction to a tissue injury. The first takes place slowly, in about 200-300 days, and it is sustained by biliary cell and mature hepatocyte proliferation; the second in some cases requires the involving of multipotent stem cells resident into liver parenchyma. In the presence of an extensive harmful stimulus, both chronic and acute, the hepatocytes proliferation alone is not enough, and the intervention of ASC is necessary.[129] The origin of ASCs, as their exact localization and mechanisms used to partecipate to regeneration have not been completely understodden yet. Many experiments on rats showed the presence of stem cells capable of generating a transient expansion complex that sustains hepatic regeneration when the classic proliferative mechanisms of hepatocytes and cholangiocytes are blocked or insufficient.[130,131] That cells, called "Oval cells", have been characterized in detail from their molecular profile in many murine models,[132] but their omologous in the human liver has never been exhaustively described.[124,133] In humans many studies showed the presence of small cells with stemness characteristics placed inside Hering's channels;[134] these cells are capable to generate hepatocytes or cholangicytes if necessary. They can switch on/off Notch pathway in response to paracrine stimulation from cells of inflammatory local microenvironment

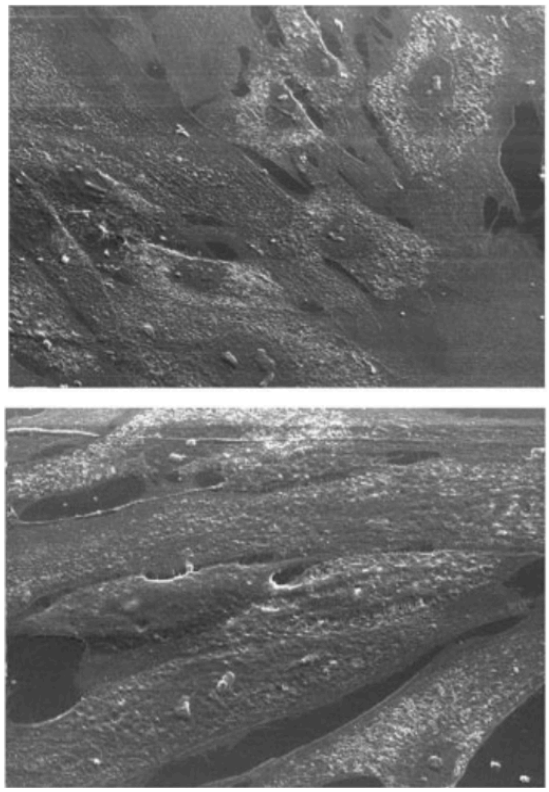


Figure 11: HLSC images obtained by electron microscopy. Adapted from "Isolation and Characterization of Stem Cell Population from Adult Human Liver, Herrera MB et al., 2006" [137]

(portal myofibroblasts if there is a biliary injury, macrophages if the injury involves hepatocytes).[135,136]

Furthermore, despite the litterature on that argument is already extensive, so far the clinical application of stem cells in liver diseases treatment is only a promising experimental approach. The exact phenotype of liver ASC and the molecular processes involved in their differentiation ar far to be unequivocally defined and accepted by the scientific community, thus other specific studies are needed.[130]

In 2006 Prof. Camussi's group isolated, from adult human livers biopsy specimens, a population of cells with ASC features (figure 11).[137] These cells, defined as Human Liver Stem Cells (HLSC), express the typical stem cells markers such as CD29, CD73, CD44 and CD90, but have in the meantime, a partial hepatocyte commitment, proved by the expression of albumine, α-fetoprotein, cytokeratine 8 and cytokeratine 18. Moreover HLSCs differ from hematopoietic stem cells because they do not express the typical hematopoietic markers as CD34, CD45, CD117 e CD133. Also they can not be identified as Oval Cells, infact they are different from them for their differentiation capability and for surface markers expressed (HLSCs are negative for CK19, CD117 e CD34). If cultured in the presence of growth factors as HGF e FGF4, HLSCs can differentiate in mature hepatocytes, able to syntetize hepatospecific molecules such as cytochrom P450, albumine and urea. This is not the only possible destiny of these cells: using specific culture media it is possible to stimulate HLSCs to produce structures similar to

pancreatic islets or to differentiate towards osteogenic or endothelial lines. The hight plasticity of HLSCs, together with their easy isolation, made these cells promising candidates for development of new cell-based therapies in the field of regenerative medicine. In the paper of Herrera HLSCs were able to regenerate liver parenchyma after acute liver failure induced by N-acetil-p-aminophenol in SCID mouses.[137]

The hepatoprotective ability of HLSCs has again been confirmed by a lethal model of fulminant liver failure in SCID mouses: the systemic administration of HLSC or their infusion with direct puncture of hepatic parenchyma led to the survival of the treated animals, to organ function recovery and to a complete morphological *restitutio ad integrum*.[138]

Furthermore, ex vivo experiments of re-cellularisation of hepatic bio-scaffolds obtained by de-cellularization of rat livers have demostrated the regenerative ability of HLSCs and their organ-specific differentiation.[139]

5.3 Extracellular vescicles

The Extracellular Vesicles (EVs) are small vescicles delimited by membrane, released by the cell in order to provide an intercellular communication and considered very important molecular carriers both in eukaryotes and in prokaryotes.
EVs secretion was initially described as a method to eliminate not necessary components from the cell, but is more and more clear that their role is not only waste carriers. Infact they transports many biomolecules (lipids, proteins and nucleic acids) and can actively participate to tissues omeostasys maintaining, in pathologic and physiologic condition.[140]

The definition “extracellular vescicles” actually includes many different cathegories of vescicles that are identified and named in accordance with their dimension, the cell of origin and the content. On the basis of current knowledge about EV biogenesis, is possible to divide them in two main cathegory: exosomes and miclovescicles (table 8 and figure 12).[141]

- Exosomes are vescicles with a diameter between 50nm and 100nm released during maturation of reticulocytes. They are intraluminal vescicles formed by internal membrane of endosome gemmation, and successively secreted by the fusion of the exosome itself with lisosomes or directly with plasma membrane.

- Microvescicles are an exocytosis product: they origin by means of an external gemmation mechanism from plasma membrane and are characterized by a diameter between 50 nm and 500 nm (they can reach 1µm in case of oncosomes).

	Exosomes	Microvescicles
Origin	Endosomes	Plasma membrane
Dimension	50-150 nm	50-500 nm (until 1 µm)
Other names (according to origin, morphology, dimension …)	- Exosome-like vesicles - Nanovescicles - Prostasomes - Tolerosomes - Dexosomes	- Microparticles - Blebbing vesicles - Shedding vesicles - Oncosomes - Apoptotic bodies

Table 8. Main differences between exosomes and microvescicles.[141]

As already anticipated, EV content includes various bioelements such as proteins, lipids, sugar and nucleic acids:

- The protein content of EVs includes a spectrum of proteins derivated from plasma membrane and cytosol. Among them it is possible to identify many adhesion molecules as CD9, CD11a, CD11c, CD18, CD146, CD166, and some *heat shock proteins,* as Hsp70 and Hsp90 that can facilitate the binding with major histocompatibility complex (MHC).[141]
- Lipids contained inside EVs are several: cholesterol, diglycerides, sphingolipids (sphingomielin I and ceramide), phospholipids, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamide, phosphatidylinositol. Also bioactive lipids can be present such as prostaglandins and leukotrienes.[142]
- Many nucleic elements are transported inside EVs, between them there are RNA, mRNA and miRNA. miRNA filaments usually are binded to Argonaute proteins and thanks to these proteins they can influence hundreds of mRNA.[143]

Once EVs are released in the extracellular space, they can interact with their target and carry out their effect by means of many modalities: by activating membrane receptors, by internalization (endocytosis) or through the fusion with plasma membrane. The activation of a cellular pathway following the binding with a

specific receptor tipically happens on follicular dendritic cells,[144] on intestinal epithelial cells,[145] on neurons, on lymph nodes, on liver cells[146] and on lungs cells. The main mediators of this interaction are integrins, lecithins, proteoglycans, heparan sulfate and extracellular matrix components. Sudies in neurological field, using EVs derived from microglia and from cells derived from neuroblasthome, demostrated that the destiny of EVs depends on the content of EVs themselves and on the type of receptors expressed on target cells.[147]

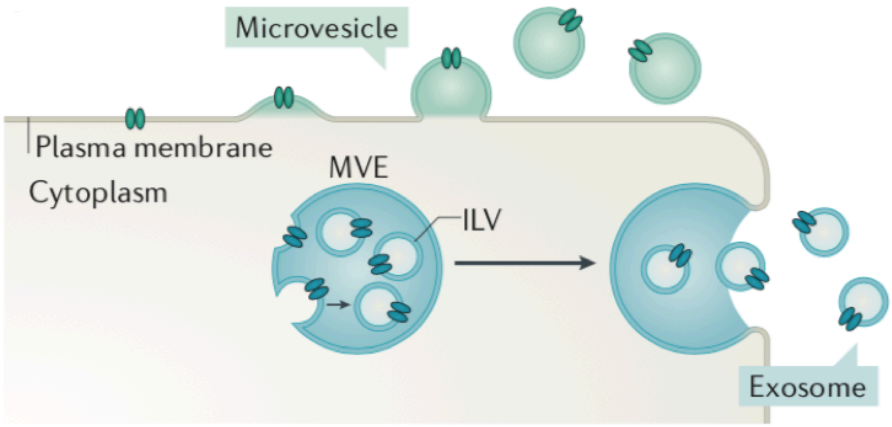


Figure 12. Gemmation of exosomes and microvescicles. Image adapted from "*Shedding light on the cell biology of extracellular vesicle*, Van Niel G et al., 2018".[141]

The EVs isolation is a difficould procedure, given their fragility and their small size, therefore in the major part of studies authors prefer to perform a purification of EVs from cell cultures rather than their direct isolation from biologic tissues or fluids. During these purification processes the centrifugal force is used to settle particles into the solution. Accelerations between 200 and 1.500g are applied in order to remove cells and cellular debris and successively from 10.000 to 20.000g to plunge vescicles greater than 100nm, while for EVs smaller than 100nm an acceleration of 100.000-200.000g is needed.[146] This protocol of ultracentrifugation has some criticisms because it does not guarantee a proper separation on the basis of dimension and shape, and, overall, because EVs, when subjected to such a high acceleration, could coalesce, brake or even activate.[148]

Many evidences show a fundamental role of EVs during both the embryo-fetal period and extra-uterine life. During embryonal developement mediators trasported by EVs are fundamental for a proper tissues organization: very important

mediators determining morphogenic gradient such as Wnt and Hedgehog are transported by EVs.[149,150] Another important role of EVs concerns the regulation of cellular polarity, that is essential for the proper functioning of ciliary structures.[151] The intercellular comunication reaches the maximum expression in the nervous system, and also in this setting the role of EVs has been studied in detail.[152]

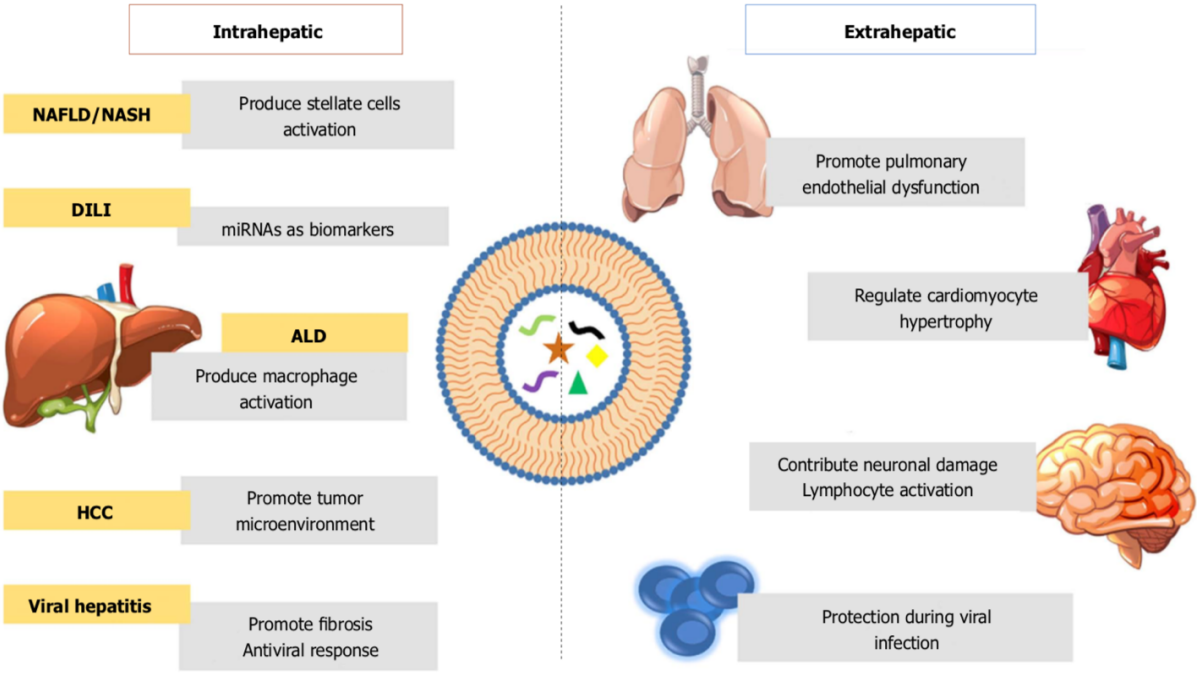


Figure 13. EVs effects demostrated at extrahepatic and intrahepatic level. Image adapted from "*Extracellular vesicles in liver disease and beyond*, Morán L et al., 2018".[153]

In parallel with their role in physiological conditions, recent studies have demostrated that EVs actively take part in mechanisms of respose to a pathological status. Among the several diseases in wich an action of EVs is documented,[154] the hepatology is a particularly interesting setting: infact the liver is a multicellular organ composed of parenchimal (hepatocytes) and non parenchimal cells (Kupffer's cells, sinusoidal endothelial cells and hepatic stellate cells) that require a sustained intercellular dialogue. The EVs released by hepatocytes have been characterized in many studies that observed that the vescicles content can vary according to the underlying disease: for example in the serum of patients affected by NAFLD or NASH there are levels of EVs significantly higher. After having characterized these vescicles, their content was analyzed and lipotoxic fatty acids and miR128-3p have

been found. These component are able to activate hepatic stellate cells inducing fibrosis.[155] Another example is rapresented by HCC, where EVs produced by neoplastic microenvironment seem to contribute to proliferation and neoplastic growth, facilitating metastatization process.[156] Further intra ed extra-hepatic effects of EVs are reported in figure 13.[153]

The therapeutic application of EVs has been studied since the last ten years, particularly about MSC-EVs isolated from bone marrow, adipose tissue, umbelical cord and placenta. During the last years many immunomodulating and antiinflammatory effects of MSC-EVs have been demostrated, throught a paracrine action with the cells with which they make contact. The effectiveness of MSC-EVs has been experimented on lab rats in many models of injury:

- Hepatic IRI
- Acute hepatic failure
- Hepatic fibrosis
- Chronic Inflammatory Bowel Diseases
- Myocardial IRI
- Acute renal injury
- Cerebral thraumatic injury and sub-cortical stroke
- Pulmonary hypertension
- Graft-versus-host disease in allogenik skin transplantation

In all these models a recovery of organ functionality was observed together with a regeneration of injuried cells, a decrease of pro-inflammation cytokines release, an increased production of anti-inflammation cytokines and an anti-apoptotic and antioxidant action.[157–159]

5.4 *Human Liver Stem Cell-derived Extracellular Vesicles*

Human Liver Stem Cell-derived Extracellular Vesicles (HLSC-EVs) are a kind of EVs with promising potential of application in the regenerative medicine field. They have been widely studied by the group of prof. Camussi in Turin.

They have an eterogeneous dimension, an average size of 174±64 nm and express typical exosomal markers, such as CD81, CD63, Alix and Hsp90, and specific markers of mesenchimal cells as CD29, CD90, CD44 and CD73.

In kidney, HLSC-EV were capable to induce the functional recovery in a murine model of acute renal failure induced by glycerol in immunodeficient animals.[160] Moreover, always in nephrologic context, they demostrated the ability to prevent fibrosis, inhibiting the up-regulation of pro-fibrotic genes (TGFβ-1, Col1a1) and pro-inflammatory cytokines.[161]

Regards the liver the effectiveness of HLSC-EVs has been largely studied on experimental models with small animals. The paper of Herrera et al.[146] demostrated that the systemic administration of HLSC-EVs in a model of partial hepatectomy in the rat, significantly improves the procedure's *outcomes*. It provided an important reduction of AST and ALT, an increased production of albumine and the rise of remnant liver weight.

Histologically HLSC-EV determined an improvement of microscopical aspect and a reduction of apoptosis evaluated by PCNA. Regarding molecular mechanisms involved, it has been shown that inside EVs are contained many factors fundamental for cellular proliferation and for protection against apoptosis (MATK, MRE11A, CHECK2, MYH11, VASP, CDK2, BCL-XL, BCL2 and BIRC8).[146,162] Again in the study of Herrera it was identified α4-integrine as a key molecule for internalization of HLSC-EV inside hepatocytes (figure 14). Recently Camussi's group led a study in vitro to evaluate the effectiveness of HLSC-EV for the treatment of Citrullinemia type 1, an autosomic recessive disease caused by a defect of the enzyme Arginine-Succinate Synthase 1 (ASS1). HLSC-EVs allowed the transferring of mRNA codifying for ASS1 and of the enzyme ASS1 itself, inside HLSCs isolated in liver biopsies taken from a patient affected by that disease,

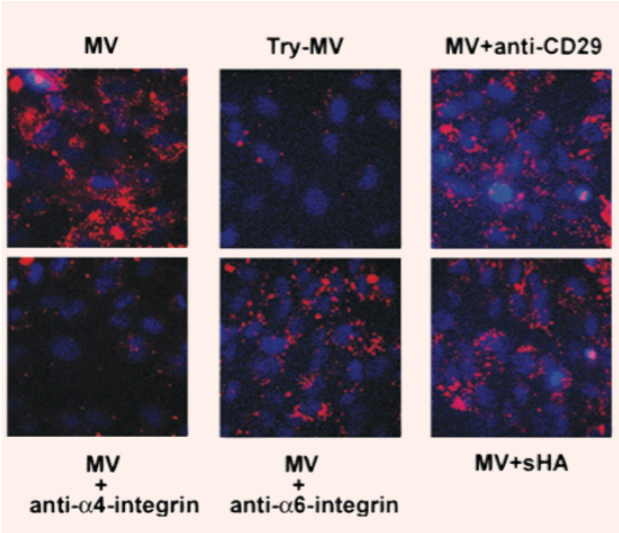


Figure 14. Images of HLSC-EV obteined with confocal microscopy: the pre-incubation of HLSC-EV with anti-α4-integrine antibidies inhibits their internalization into the hepatocyte. Adapted from "*Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats*, Herrera MB et al, 2010".[146]

leading to the restoration of ASS1 activity and urea production.[163] Finally, in the already mentioned paper of our group,[119] we demostrated in an hypoxic model of NMP, that HLSC-EV are able to integrate into hepatic parenchyma in 4 hours of perfusion, as confirmed by the epifluorescence microscopy images. Livers subjected to hypoxia during NMP have taken advantage from the treatment with HLSC-EV reducing AST and LDH release and showing a significant reduction of apoptosis and necrosis in biopsies taken at the end of 4 hours perfusion.

Between the advantages of the clical use of HLSC-EVs in the setting of regenerative medicine, there is the fact that they share in different damaged tissues the same protective action of the tissue of origin, without having the technical problems linked with the therapeutic use of stem cells (immunity and rejection). Furthermore HLSC-EV can be easily stored at -80°C and used within 6 months from their production without losing their effects. Considering this, it is evident than the use of HLSC-EV is more similar to a pharmacological treatment than a stem cell therapy.

6. EXPERIMENTAL STUDY

6.1 Backgrounds

Liver transplantation, the only effective therapy for the majority of terminal hepatic diseases, is affected by a great gap between the number of patients candidates for the procedure and the available organs.[12] In order to reduce this limiting factor, there are new techniques in course of evaluation aimed at expanding donor pool.[80] In particular, many transplantation centers are trying to expand their experience in using Extended Criteria Donors (ECD), by accepting for transplantation donors affected by hepatic steatosis, or old donors or also donors deceased for cardiac death. ECD organs allow a significant increase of number of available livers, [44] but they are associated with a worsening of post-OLT results in terms of PNF, EAD, graft and patient survival.[56,64]

The gold standard for liver preservation today is represented by Static Cold Storage (SCS); however this method, when used for preserving sub-optimal livers, is less effective in protecting the organ from IRI.[40] Considering the augmented sensitivity of ECD livers to ATP depletion during cold ischemia,[48] ex vivo normothermal perfusion system can be a valid alternative for preserving this kind of grafts. Normothermal Machines Perfusion (NMP), infact, are disposables able to deliver oxygen and nutrients to hepatocytes, maintaining the organ at physiological temperature (37°C).[164] During normothermal perfusion liver cells are metabolically active, as demostrated by bile production, lactate clearance and glucose consumption.[110]

Many studies have demostrated the feasibility and the increased effectiveness of NMP in preserving organs from DCD donors,[106]however, NMP alone is not capable to ricondition hight risk organs, that currently are considered not safe enought and are discharged for transplantation.[44] On the other hand NMP offers the possibility to pharmacologically treat the graft during perfusion, considering that normothermia allows a normal metabolic activity of hepatocytes.[116]

HLSCs are multipotent stem cells isolated from liver parenchyma of human adults, that express hepatic markers such as albumine, α-fetoprotein and cytokeratine

(8 and 18).[137] Many studies demostrated their organ-specific differentiation and their hepatoprotective and regerative power.[138,139,163] HLSCs implement their biologic activity also by paracrine mechanisms: it was described that they can release HLSC-EVs into a conditioned culture medium. These are small vescicles equipped with membrane and containing lipids, proteins and genetic material (mRNA, miRNA) involved in cellular proliferation and tissue regeneration. HLSC-EV, infact, allow the communication between a stem cell and an adult cell by the horizontal transfer of genetic informations, that are then effectively translated into proteins characterized by biological activity.[154,162] It is interesting to notice that, in experimental conditions, HLSC-EVs have demostrated to share the majority of the protective effects specific of the cells from which they derives.[146,160]

Recently we used HLSC-EVs for reconditioning of organs subjected to hypoxia during NMP.[119] On the basis of these preliminary results, it is interesting to notice that the combination NMP and HLSC-EVs results effective in treating DCD livers, in a model able to simulate as closely as possible the clinical reality of hight risk donation.

6.2 Materials and methods

Study design

All the described experiments have been carried out in the Molecular Biotechnology Centre of Turin in Prof. Camussi's lab.

First of all it was performed a series of preliminary tests intended to perfect the experimental model. In particular the goals of this first phase were the following:

- Setting up of the surgical procedure of hepatectomy and liver transport
- Optimization of NMP circuit
- Evaluation of the warm ischemia model as a surrogate of DCD donation

Once the model was standardized, the animals were divided in the following experimental groups:

- NO INJURY group: including 6 livers procured trying to limit the warm ischemia time to a minimum and trasported from the enclosure to the perfusion chamber rapidly and in hypothermic conditions, in order to guarantee a virtually

absent tissue distress. The organs were then perfused in the NMP circuit for 6 hours.

- WARM ISCHEMIA group (WI): including 6 livers subjected to 60 minutes of controlled warm ischemia, to mimic the tissue injury of DCD donation. The organs were then perfused in the NMP circuit for 6 hours.
- WARM ISCHEMIA + EV1 group (WI+EV1): including 6 livers receiving 60 minutes of controlled warm ischemia and treated during NMP with HSLC-EV at a lower dose. The organs were perfused in the NMP circuit for 6 hours.
- WARM ISCHEMIA + EV2 group (WI+EV2): including 6 livers exposed to 60 minutes of controlled warm ischemia and treated during NMP with HSLC-EV at a higher dose. The organs were then perfused in the NMP circuit for 6 hours.

To reproduce DCD donation we choose 60 minutes of warm ischemia on the basis of what described in the litterature: the majority of experimental works about MP for ischemic liver reconditioning had used the same timing.[85,104] That choice is justified by the fact that the DCD graft associated with a significant worsening of post-OLT outcomes are that subjected to a WIT of at least 45 minutes.[38]

The study has been ideated in order to confirm the effective ex vivo interaction between stem cells derived products and rat hepatocytes in an experimental model of normothermal perfusion of DCD livers, and evaluate the biologic effects of the treatment with HLSC-EVs on hepatic functionality and morphology during organ preservation.

Housing of animals

For our research it was not possible to adopt scientifically valid methods alternative to animal experimentation, such as in vitro tests or computer modeling, because this kind of study has the object of evaluate biologic interactions of HLSC-EV in the NMP setting, in order to consider their subsequent use on humans. Human experimentation indeed is not applicable, for ethical reasons, without preclincal studies.

Animal studies have been authorized by the Ethics Committee of National Institute of health (Protocols n°1164/2015-PR and n°262/2019-PR) and conducted in accordance with the National Institute of Health Guide for Care and Use of

Laboratory Animals. 24 male Wistar rat aged 8 to 12 weeks have been used (species *Rattus Norvegicus*) (200-250g weight). All these animals have been obtained from *Charles Rivers*-Italy and housed in the enclosure of Molecular Biotechnology Centre of Torino in standard conditions of lighting and environmental temperature and free access to water and food.

Surgical procedure

In the rat the liver is placed inside the abdominal cavity in right subdiaphragmatic place. It has, as a special characteristic, four different well recognizable lobes, separated from each other by deep scissures (median lobe, caudate lobe, right lateral lobe, left lateral lobe); the gallbladder is absent. Hepatic hilum is placed outside the liver, and it curves with concavity towards left at its entrance inside the liver parenchyma. The pedicle is completely covered by a peritoneal ligament that continues with hepatic capsule. In the pedicle can be identified:

- The common bile duct (CBD), that split inside the liver in a left and a right branch;
- The hepatic artery (HA) that is placed under the bile duct but above portal vein and follows the same distribution of bile ducts;
- The portal vein (PV) that is the deepest structure of hepatic pedicle. It splits inside the liver too, in three main branches; one for the caudate and median lobe, one for the right lateral sectors, and the tird for the left lateral sectors.

The hepatic veins (HVs) are divided in inferior HVs that drain caudate lobe and right lateral segments, and superior HVs that are formed by a right branch, a median branch and a left branch and drain respectively right, median and left sectors.

The operative field is composed by a stereoscopic microscope, a plexiglass board and by surgical instruments: a needle-holder, two right forceps, two angled microforceps, two scissors (curved and right), one kelly forceps, an Abbocath-T Cannula of 18G (green), and an Abbocath-T Cannula of 22G (blue), silk 6/0 and 4/0 sutures, gauzes, syringes filled with saline solution or Celsior (figure 15).

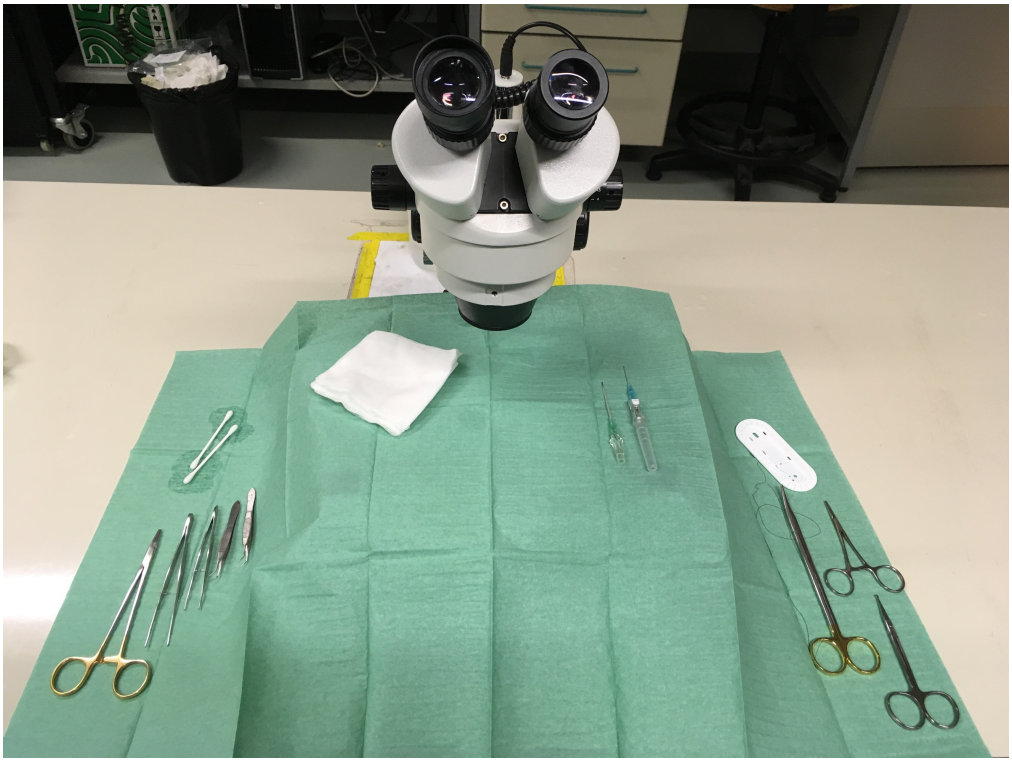


Figure 15. The operative field.

The whole surgical procedure took place after narcosis induction. The general anaesthesia is obtained by intramuscular administration of 80mg/kg of Zolazepam+Tiletamine (*Zoletil*®) and 16mg/kg of Xilazine (*Rompun*™). The confirmation of complete sedation of the animal is obtained by the application of a pain stimulus on the tail.

The surgery starts with intraperitoneal heparin administration (1000-1500 UI) and skin disinfection. Then a midline laparotomy is performed. The liver is mobilized with the section of falciform ligament and the liver parenchyma is protected by a gauze, the suprahepatic inferior vena cava segment and hepatic veins can be identified. These are legated by a silk 6/0 suture (figure 16A and 16B). The bowel is irrigated with saline and retracted to expose the liver and the hepatic pedicle. The main structures are identified, in particular the CBD and the PV. We proceed with gentle dissection of CBD and its surrouding with two silk 4/0 laces (figure 16C). The CBD is punctured and cannulated as distal as possible, using an Abbocath-T 22-G cannula (figure 16D and 16E). Both the silk laces are ligated in order to firmly secure the cannule to the CBD (figure 16F and 16G). The following steps are the PV

isolation and dissection and its surrounding with silk 4/0 laces (figure 16H). The PV is punctured near its origin and cannulated with an 18-G cannula (figure 16I), that is blocked by the two silk 4/0 laces (figure 16J). At the moment of PV cannulation 80% of the liver blood flow is stopped; for this reason from this moment the warm ischemia starts.

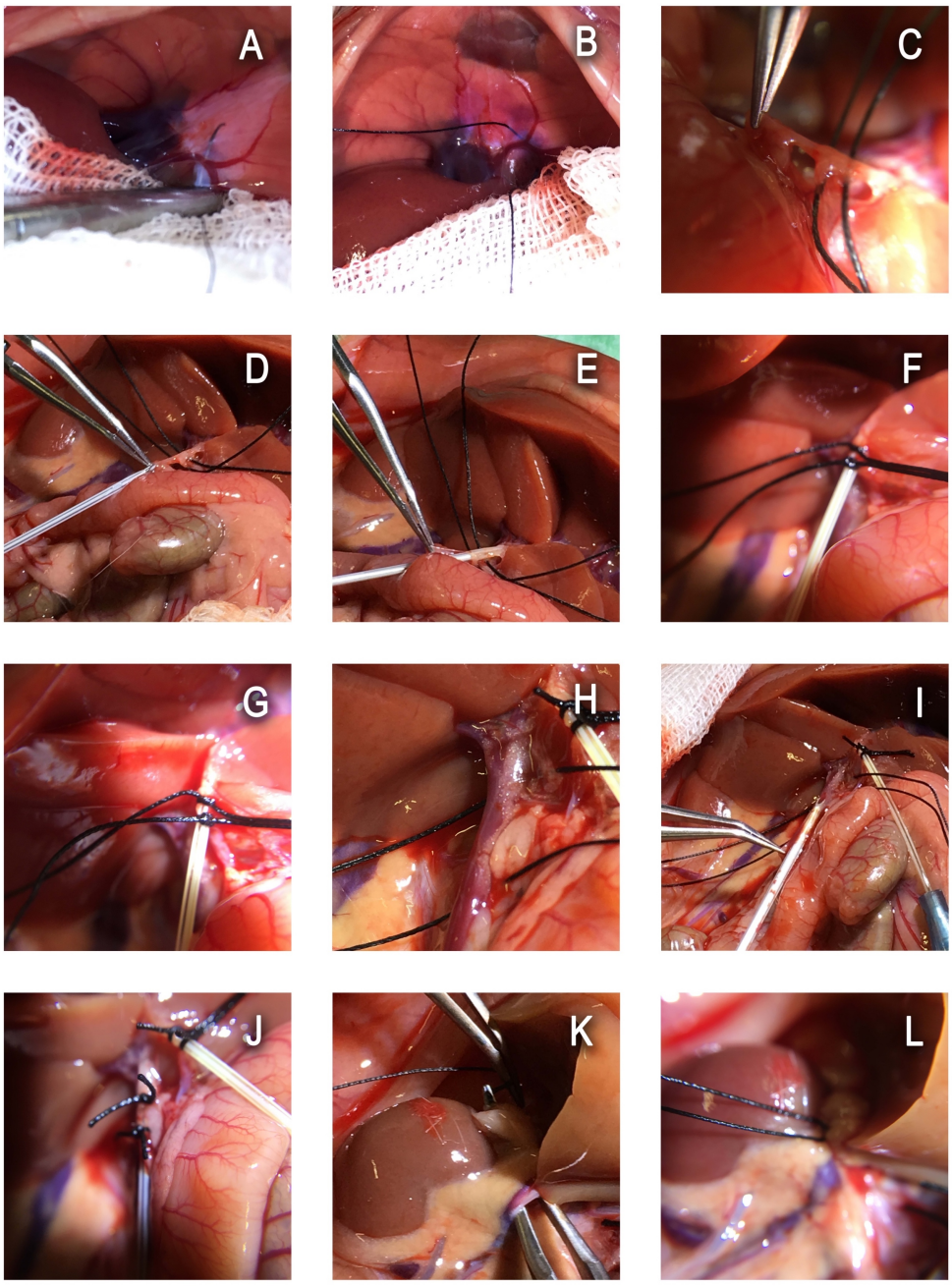


Figure 16. Total hepatectomy surgical procedure. [A, B] Diaphragmatic veins ligation. [C] Isolation of CBD. [D, E] Cannulation of CBD. [F, G] Fixation of the bile cannula. [H] Isolation of PV. [I] Cannulation of PV. [J] Fixation of portal cannula. [K] Isolation of inferior VC above the origin of right renal vein. [L] Ligation and section of sub-hepatic inferior VC.

The warm ischemia time should be reduced at minimum in the model of standard donation, hence, only in the NO INJURY group procedures the abdominal cavity at this moment is filled with ice. For testing the correct cannulation of PV and the absence of atypical resistances the liver is slowly flushed with 10 cc of cold Celsior solution (NO INJURY group) or room temperature saline solution (WI, WI+EV1 e WI+EV2 groups). It follows ligation and section of HA with consequent complete stop of hepatic blood supply. Then the subhepatic VC is ligated (figure 16K and 16L). After diaphragm section, the remainig hepatic ligaments are gently cut to free all the lobes, the inferior VC is sectioned at supra-hepatic and sub-hepatic level and the liver is removed from the abdominal cavity.

The organ is placed into a Petri dish and weighted. The transport can take place in two different modalities on the basis of the experimental groups:

- NO INJURY group: the liver is immersed in Celsior solution and the Petri dish is completely surrounded by ice, in order to closely simulate the SCS preservation; it is then transported to the perfusion room as soon as possible to limit at minimum the cold ischemia time.
- WI, WI+EV1 e WI+EV2 groups: the Petri dish where the liver is placed is filled with saline previously rewarmed at 37°C.

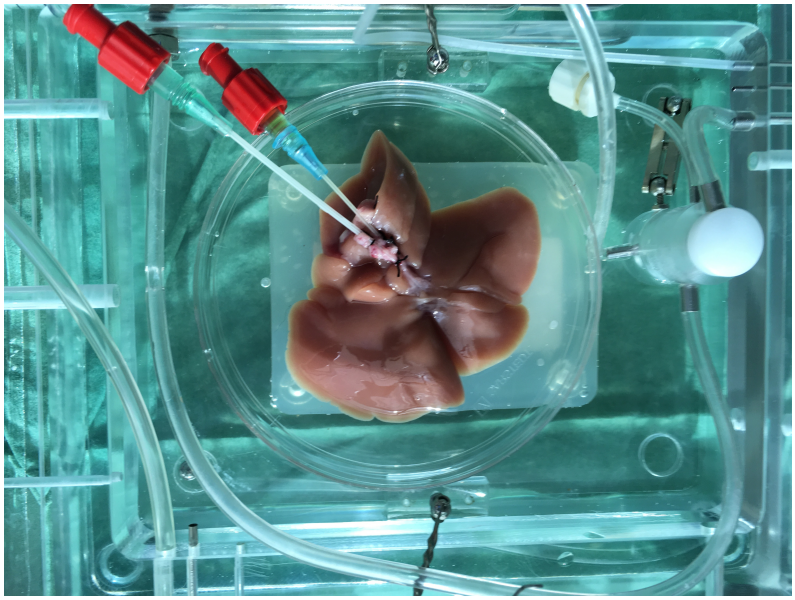


Figure 17. DCD liver maintained in controlled warm ischemia condition for 60 minutes.

In the perfusion room if the procedure is one of the NO INJURY group the liver undergo a slow portal flush with 10-15 cc of Williams-E-Medium and immediately connected to the perfsion circuit, completing the ischemia time at this point. The livers of the remaining three groups are maintained in a controlled temperature condition (37°C) till the end of the 60 minutes of warm ischemia time. After this period, the organ is flushed with 10-15cc of Williams-E-Medium and connected to NMP.

NMP perfusion system

The perfusion circuit was assembled following a standardized model described by University of Harvard's group,[100] by making the necessary modifications useful to guarantee an adequate perfusion for our setting.[165] As in all the NMP systems, the circuit is composed by the following components (figure 18):

- Perfusion Chamber (*Moist Chamber - Harvard Apparatus, Hugo Sachs Elektronik*): it is a plexiglass chamber, inside which the liver is placed. There are two metal cannulae, one is connected to the portal cannula and the second is used to suck the perfusate that flows freely into the chamber from the VC. In the core of the container there is a serpentine in which the perfusate flows in contact with the water rewarmed by the heat exchanger. It allows to keep the temperature of the perfusate stable at 37°C. A small bubble trapper is placed immediately before the portal inflow to prevent the formation of air emboli.
- Reservoir: a 200 ml becker is used as a reservoir to collect the circulating perfusate. A 40 μm Nylon filter (*Falcon*) is placed on the superior part of the reservoir in order to avoid clots circulation.
- Oxygenator: for the perfusate oxygenation we used an hollow fiber oxygenator connected with a tank containing a mixture of O_2 at 99% and a valve. The gas inside the oxygenator diffuses through fibers membranes and dissolves in the perfusate. Oxygen flow is set in order to guarantee a $pO_2 > 400$ mmHg in the perfusate, necessary to support the metabolic needs of the liver tissue during NMP.[166]
- Bubble trapper: other than the bubble trapper placed in the perfusion chamber, there is another one just after the oxygenator, to remove air emboli from the

perfusate, eventually produced by the oxygenator itself. This trapper is designed also to induce the Windkessel effect, that allows a trasformation of the pulsatile flow produced by the pump into a continuous flow.

- Amplificator for the flow and pressure control (PLUGSYS Modular Measuring & Control System - Harvard Apparatus, Hugo Sachs Elektronik): it is a disposable that automatically adjusts the flow in order to guarantee a costant perfusion pressure preset by the operator. Based on Darcy's law (Flow = ΔPressure/Resistance), perfusion circuits can be grossly divided in two categories: controlled flow circuits and controlled pressure circuits. In our model we chose the last option because in the controlled flow systems pressure can increase abruptly causing damage to the hepatic micromicrocirculation.[166] The amplifier is formed by two modules: the Transducer Amplifier Module (TAM-D) receives the signal from a pressure trasducer connected to the portal cannula, while the Servo Controller (SCP Type 704) establishes the flow by regulating the rotation speed of the pump to which it is connected. To provide a proper flow to the liver, the perfusion pressure has been regulated on the organ weight within 10 and 16 mmHg.
- Pumps: two peristaltic pumps (REGLO Analog Peristaltic Pump - ISMATEC) have been used for the circulation of the perfusate. One pump, connected to the modular amplification PLUGSYS, provides the inflow to the liver, while the other allows the aspiration of the perfusate that flows from the VC collected in the Petri's dish.
- Warming system: it consists in a second circuit linked in paralel to the perfusion chamber that allows to maintain the perfusate at a constant temperature of 37°C. The water contained inside this is rewarmed and circulated by a thermocirculator (*LAUDA Heating Thermocirculator*).
- Continuous infusion: during the perfusion two infusion pumps (Terufusion® Syringe Pump - TERUMO) connected to the circuit provide continuous addiction of bile salts, insuline and nutrients to the perfusate.
- Tubes and sampling system: the whole circuit is assembled with flexible tubes in Tygon®, used to connect the described components. Before and after the perfusion chamber are placed two 3-way taps; one is used for HLSC-EV

administration and for taking samples of the perfusate that flow towards the liver, the second permits to sample the perfusate that comes out from the liver.

At the end of every experiment the circuit is completely washed and cleaned with the circulation of about 1 L of distilled water followed by NaOH 0,5N (about 250 ml), and again distilled water to remove the excess of NaOH.[167] Tubes are then further washed separately and dried. The oxygenator is cleaned with distilled water (about 2 L), that circulate slowly inside overnight by means of a dedicated peristaltic pump. After 10-12 uses the oxygenator is replaced. Before every experiment the circuit is assembled *ex novo*.

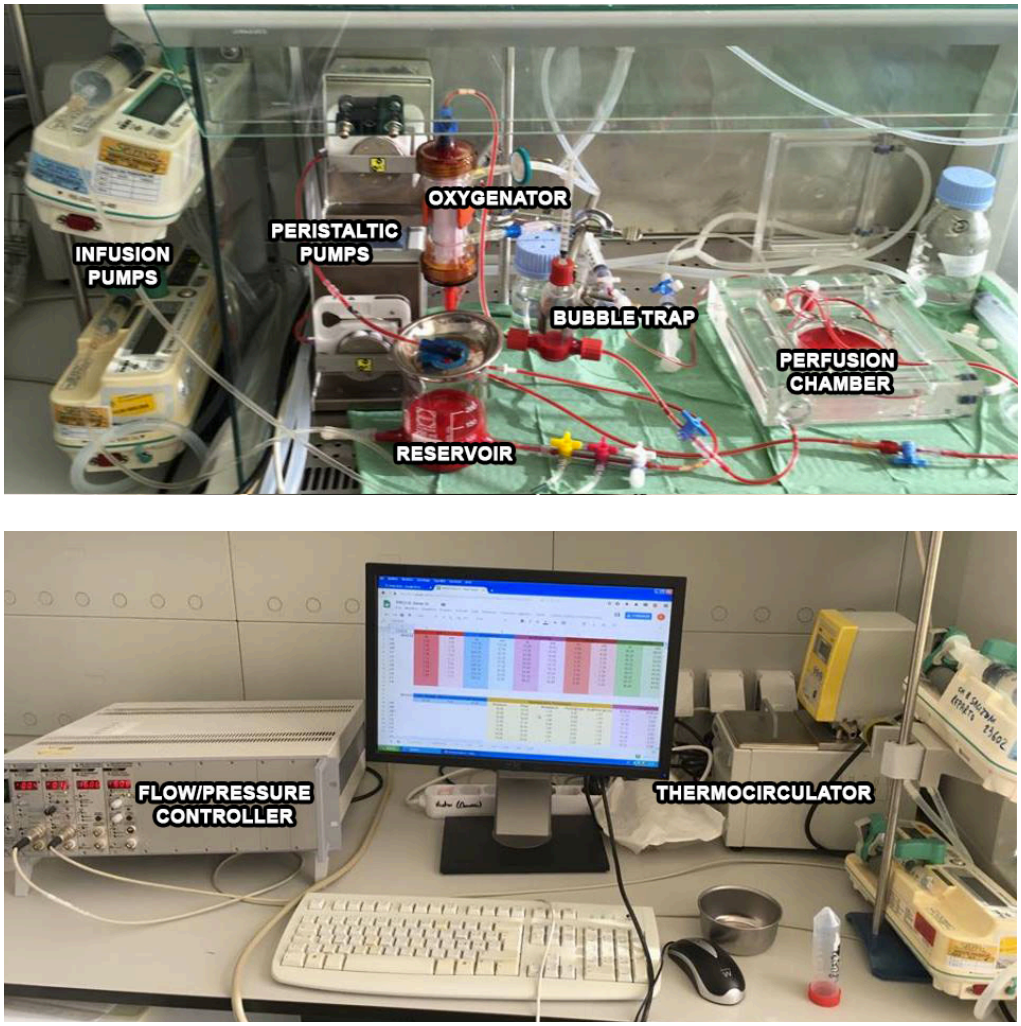


Figure 18. NMP circuit components

The perfusate

For choosing the perfusate we followed the litterature[100] and our experience in the lab.[119] In particular, to guarantee a good priming of the circuit, we chose a volume of 150 ml, composed by 100 ml of Williams E Medium and 50 ml of human red blood cells.

Williams E Medium, is a culture medium specific for hepatocytes containing organic salts, amino acids and vitamins. We added 100 UI/ml of penicillin (*Sigma-Aldrich*®) and 100 μg/ml of streptomycin (*Sigma-Aldrich*®) to prevent the bacterial contamination of the system, 0,292 g/L of L-glutamine (*Sigma-Aldrich*®) that is absent in the medium formulation, 1 UI/ml of insuline (*Lilly*, Italia) to stimulate the hepatic uptake of glucose, and 1 UI/ml of heparin (*PharmaTex*, Italia) to avoid the formation of clots.

The packed red blood cells were donated by the S.C. Banca del Sangue e Immunoematologia (A.O.U. Città della Salute e della Scienza di Torino) thanks to the collaboration with Dr. Daniela Bassino. In particolar the red blood cells come from expired blood bags, so not usable in clinics, but containing still viables cells. The bags are stored at 4°C and used within 5 days from the delivery. The presence of a molecular carrier of O_2 in the perfusate is fundamental to guarantee a proper liver oxygenation;[168] for this reason we used a red blood cells volume (50 ml) enough for obtaining an hematocrit of 16-20%. That value, according with the litterature,[100,104,106] allows an effective oxygen supply to the liver without increasing too much the perfusate viscosity. During the perfusion, 10 ml of red blood cells were further added if an hematocrit lower than 16% is reached.

During perfusion we decided to supplement the perfusate with a continuous infusion system, simulating what happens in NMPs for clinical use.[107] In particular we used two perfusion pumps loaded with syringes, one of 50 ml and one of 10 ml:

- 50 ml syringe: 30 ml of Williams E Medium supplemented as described before, except for heparin, that was added at the concentration of 4 UI/ml. That infusion guarantees a continuous nutritional support to the liver during perfusion, prevents clots formation keeping an high heparin concentration and helps glucose uptake thanks to insulin. The decision to infuse insulin continuously is based on the evidence of a better effectiveness of this modality compared to the bolus

administration (figure 19). The pump speed was set at 5 ml/h.

- 10 ml syringe: 60 mg of Thaurocholic Acid (*Sigma-Aldrich*®) dissolved in 6 ml of distilled water. That concentration, similarly to what described by Schlegel *et al.*,[167,169] supports the production of bile during the perfusion. The pump speed was set at 1 ml/h.

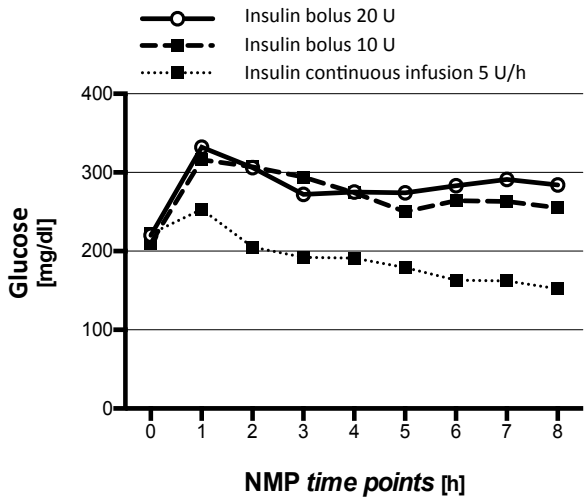


Figure 19. During the standardization phase of the model, a better efficacy of the continuous infusion of insulin in promotion of hepatic glucose uptake was observed.

Experimental protocol of NMP perfusion

Before connection of the liver to the circuit, the perfusate circulates inside the circuit and is oxygenated until the pO_2 is over 400 mmHg;[166] 3-4 mEq of NaOH are added to the perfusate to reach a pH between 7,30 e 7,50.

At the start of perfusion, the flow is adjusted manually at 1-2 ml/min, to evaluate the homogeneity of parenchima perfusion and the absence of flow obstruction; then the portal flow is settled within 10 and 16 mmHg (proportionally to the liver weight) and at that point we switch to the modality of autonomous control of PLUGSYS, surveilling constantly the absence of obstacles to the flow. The infusion pumps hence are started at the speed of 1 ml/h (10 ml syringe) and 5 ml/h (50 ml syringe). The bile cannula is connected to a thin drainage tube that drains inside an Eppendorf tube of 1,5 ml. HLSC-EV are administrated by a 1 ml syringe within 15 minutes from the beginning of perfusion after the sample passing throught a stirrer centrifuge (Vortex - VWR) to prevent EVs sedimentation.

Isolation and culture of HLSCs

HLSCs have been produced by the standard protocol described.[137] More in detail, HLSCs have been isolated by cryopreserved human hepatocytes (*Lonza Bioscience*) and cultured in culture medium α-MEM/EBM-1 (α-minimum essential medium and endothelial cell basal medium-1) with a proportion 3:1, supplemented with L-glutamine (2 mM), penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% of fetal calf serum (FCS). Cells are maintained into an incubator at 37°C, in a humidified atmosphere containing 5% of CO_2, for all the experiments were used HLSC from passage 5 to passage 8.

Isolation and characterization of HLSC-EV

HLSCs, once reached 80% of confluence, have been cultured overnight in presence of RPMI medium (Roswell Park Memorial Institute) deprived of the serum. Subsequently, the conditioned medium taken from HLSC has been centrifuged at 3500 rpm for 10 minutes to remove cellular debris and then ultracentrifuged at 100.000 rpm for 2 hours at 4°C to extract EVs. HLSC-EVs obtained have been re-suspended in RPMI medium in the presence of 1% of DMSO (dimethyl sulphoxide) to preserve them at -80°C until their usage. HLSC-EVs have been quantified and characterized using the instrument *NanoSight LM10*. To produce marked EVs we used HLSC previously labeled in vitro with the dye Vybrant DIL (1,1'-Dioctadecyl-3,3,3',3'- Tetramethylindocarbocyanine Perchlorate). To carry out a dose-effect evaluation two different doses of HLSC-EVs have been used:

- WI+EV1 group = 5 x 10^8 HLSC-EV / g of liver
- WI+EV2 group = 25 x 10^8 HLSC-EV / g of liver

Perfusate analysis

Perfusate samples have been collected by a syringe of 1 ml through the two inflow and outflow taps of the circuit, following this timing:

- t0 = sampling before perfusion starting, to have a basal landmark of the analyzed paramenters
- t1, t2, t3, t4, t5 e t6 = sampling performed every 60 minutes to obtain a

monitoring of the analyzed paramenters in hourly intervals

Each sampling consists in the aspiration of 1 ml from the proximal tap, for the blood gas analysis of portal inflow, and of 3 ml from the distal tap, used both for the blood gas analysis of the outflow and for biochemical analysis.

The blood gas analysis have been performed by an automatic analyser (*ABL 725 – Radiometer Medical ApS*) to evaluate modifications of pH, pO_2, pCO_2, hemoglobin (Hb) ed hematocrit (Ht).

The biochemical analysis, have been carried out in S.C. Biochimica Clinica – Baldi e Riberi Lab (A.O.U. Città della Salute e della Scienza di Torino) by Dr. Paola Caropreso. At the end of the experiment the samples have been centrifuged for 10 minutes at 3500 rpm to separate cells from serum, the latter have been stored at -80°C. At the moment of the delivery to the analysis lab, the serum were defrosted, again centrifuged to remove any possible sedimentation, and then transported to the lab. The biochemical analysis have been performed in an automated way (Cobas 8000 – HITACHI Roche) by measuring the concentration of surrogate markers of liver damage (AST, ALT and phosphate).

Bile analysis

A thin tube of PTFE, connected to the bile cannula, has been used for the hourly collection of bile inside Eppendorf tubes of 1,5 ml. On the obtained samples a quantitative evaluation was performed, weighting each sample by an analytic balance, and a quantitaltive evaluation too, measuring pH by a digital probe (*Mettler Toledo*).

Hemodynamic analysis

To monitor hemodynamic parameters, the values of flow and pressure registered by the PLUGSYS have been recorded every 60 minutes. The total resistance was calculated using the following equation:

Resistance = Pressure (mmHg) / Flow (ml/min).

Histological analysis

At the end of 6 hours of perfusion, multiple biopsies were performed to evaluate the hepatic injury in the different groups. In particular, a bioptic sample was temporarly preserved in saline solution (5-10 minutes maximum) and than included in OCT for the following epifluorescence analysis, while the remaining samples were fixed in formaline at 10% and than included in paraffin.

The HLSC-EVs uptake has been evaluated by epifluorescence analysis. The sections of hepatic parenchima, after 3 washing in PBS (Phosphate Buffered Saline), have been permeabilized for 5 minutes at 4°C using Triton X-100 at 0.5%, and then again washed in PBS and incubateded for 1 hour at room temperature with bovine serum albumine 3% in PBS (*Sigma-Aldrich*®). The cells nuclei have been labeled with DAPI (*4',6-diamidino-2-phenylindole*). All slides of the tissue are been assembled with Fluoromount and the images have been acquired using Cell Observer SD-ApoTome laser scanning system (*Carl Zeiss*®).

For the evaluation of parenchimal injury a labeling has been performed using Hematoxylin-Eosin (H&E) following the conventional way. On H&E slides the morphological damage was quantified using Suzuki's score,[170] a scoring system that gives a value from 0 to 4 for the three most characteristic patterns of IRI, that are edema, vacuolization and necrosis (table 9). For each tissue the assigned evaluation results from the addition of scores attributed to each item.

To quantify cellular apoptosis, a specific labeling was performed (terminal deoxynucleotidyl transferase dUTP nick-end labelling - TUNEL) that allows to find the fragmented DNA from caspases action. After a first digestion of the sliceses through proteinase-K (1 mg/ml) (*Invitrogen, Thermo Fisher*), the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (*Millipore*) was used, respecting productor's protocol. To find the cells positive for TUNEL, 3,3'-diaminobenzidine (DAB) was used (*Invitrogen, Thermo Fisher*) as a substrate for peroxidase. Finally, to obtain a background labeling, the slides have been immersed in Hematoxylin for 30 seconds. Cells positive for TUNEL have been counted blindly, using the software *ImageJ,* on 10 microscope fields representative of of the tissue, acquired with optical microscope (*Olympus BX41 - Life Science*) at a 200× magnification.

Score	Congestion	Vacuolization	Necrosis
0	Absent	Absent	Absent
1	Minimal	Minimal	Single cells
2	Mild	Mild	30%
3	Moderate	Moderate	60%
4	Severe	Severe	>60%

Table 9. Suziki's Score criteria. Adapted from "*Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine*, Suzuki S et al, 1993".[170]

Cellular proliferation has been studied using PCNA labeling (Proliferating Cell Nuclear Antigen), an immunohistochemistry technique that allows to emphasise cells nuclei in late G1 and S phase of cellular cycle. The protocol includes a first inhibition of tissue peroxidases with H_2O_2 and a following tissue incubation with a citrate buffering solution for 20 minutes at 102,5°C, in order to free antigenic sites of antibody binding. The histologic sections were then incubated for 1 hour at room temperature with primary antibody anti-PCNA (*Santa Cruz Biotechnology*), diluted in PBS-BSA 0,1% with 1:300 ratio. After 3 washing in PBS-Tween-0,1%, a further incubation of 1 hour at room temperature was done using the secondary antibody (*Horseradish Peroxidase Antibody*), it diluted too in PBS-BSA 0,1% with 1:300 ratio. The histologic sections are further washed with PBS-Tween-0,1% solution. To mark the positive cells for PCNA antigen, DAB was used. Finally, to obtain a background colouration, slides were immersed in Hematoxiline for 30 seconds. For every slide 10 representative photos were taken using a light microscope (*Olympus BX41- Life Science*) at a magnification of 200×. On these micrographs we performed the manual blind count of PCNA positive and negative cells using the software *ImageJ*. The proliferation index was obtained by the ratio between the number of positive cells and total number of cells, the result is expressed in percentage.

Statistical analysis

The data were described using mean and standard error. The normality of the groups has been evaluated using the D'Agostino-Pearso's, Shapiro-Wilk's e Kolmogorov-Smirnov's tests. Statistical analysis were conducted using one or two ways ANOVA, or Kruskal Wallis' test, depending on the type of data; the *post-hoc* tests were

performed by *Tukey's multiple comparison test* or *Dunn's multiple comparison test* depending on the presence of parametric variables (*GraphPad Prism*, version 6.00). We considered the results statistically significant if the P *value* < 0.05.

6.3 Results

Standardization of the model and homogeneity of the study groups

In order to evaluate the effectiveness of the treatment with HLSC-EVs in the normothermic perfusion setting of DCD livers the study was organised into four experimental groups: a control group with organs not subjected to damage (NO INJURY), a control group with organs injuried by warm ischemia time (WI) and two groups subjected to warm ischemia and treated with HLSC-EV at different doses (WI+EV1 e WI+EV2).

As shown in table 10, the organs of NO INJURY group were subjected to very few minutes of warm ischemia and to only about 20 minutes of cold ischemia (necessary for the organ transport to the perfusion room). That timing ensured a minimal liver injury, allowing to obtain the same parameters of function during NMP described in litterature. In the other three groups we applied an experimental model of DCD donation, keeping the liver at 37°C for 60 minutes.

All the groups resulted to be homogeneous regarding operative parameters during perfusion, there were no statistically significant differences in terms of weight, perfusate oxygenation, hematocrit, perfusion pressure and pH (table 10).

PARAMETER	NO INJURY Median (SEM)	WI Median (SEM)	WI+EV1 Median (SEM)	WI+EV2 Median (SEM)	p value
Number	6	6	6	6	-
Ischemia [min] **- cold** **- warm** **- total**	 3,00 (1,00) 33,8 (7,34) 36,8 (8,24)	 - 60,0 (0) 60,0 (0)	 - 60,0 (0) 60,0 (0)	 - 60,0 (0) 60,0 (0)	-
Liver weight [g]	17,10 (1,93)	15,17 (0,83)	14,17 (1,28)	13,67 (1,17)	**0,40**
pO_2 [mmHg]	500,9 (32,1)	448 (39,6)	484,6 (38,3)	517,9 (25,6)	**0,61**
Hematocrit [%]	19,02 (0,47)	17,97 (0,50)	18,56 (0,60)	18,47 (0,62)	**0,47**

Portal cannula pressure [mmHg]	15,69 (0,02)	15,19 (0,13)	14,83 (0,14)	14,98 (0,26)	**0,36**
pH					**0,23**
- t = 0 min	7,35 (0,05)	7,44 (0,08)	7,50 (0,05)	7,41 (0,03)	
- t = 60 min	7,24 (0,05)	7,26 (0,08)	7,28 (0,02)	7,32 (0,05)	
- t = 120 min	7,30 (0,05)	7,24 (0,06)	7,37 (0,09)	7,30 (0,04)	
- t = 180 min	7,33 (0,05)	7,24 (0,06)	7,39 (0,05)	7,36 (0,03)	
- t = 240 min	7,30 (0,07)	7,24 (0,05)	7,44 (0,07)	7,34 (0,05)	
- t = 300 min	7,28 (0,05)	7,23 (0,06)	7,38 (0,04)	7,35 (0,06)	
- t = 360 min	7,23 (0,06)	7,32 (0,09)	7,27 (0,06)	7,26 (0,04)	

Table 10. Operative parameters.

Internalization of HLSC-EVs into liver tissue

For verifying the actual integration of HLSC-EV we used vescicles marked with Vybrant DIL fluorophore (*1,1'-Dioctadecyl-3,3,3',3'- Tetramethylindocarbocyanine Perchlorate*) and an epifluorescence analysis was performed on tissues at the end of perfusion. By means of this labelling it was possible to evidence HLSC-EVs membrane in red and parenchimal hepatic cells nuclei in blue, labeled using DAPI (*4',6-diamidino-2-phenylindole*). As shown in figure 20, in WI+EV1 and WI+EV2 groups we could observe the presence of EVs (in red) inside the tissue cells. This analysis confirmed the possibility to vehiculate HLSC-EV treatment throught NMP circuit and demonstrated the ability of these EVs to settle inside hepatic parenchima within 6 hours of perfusion.

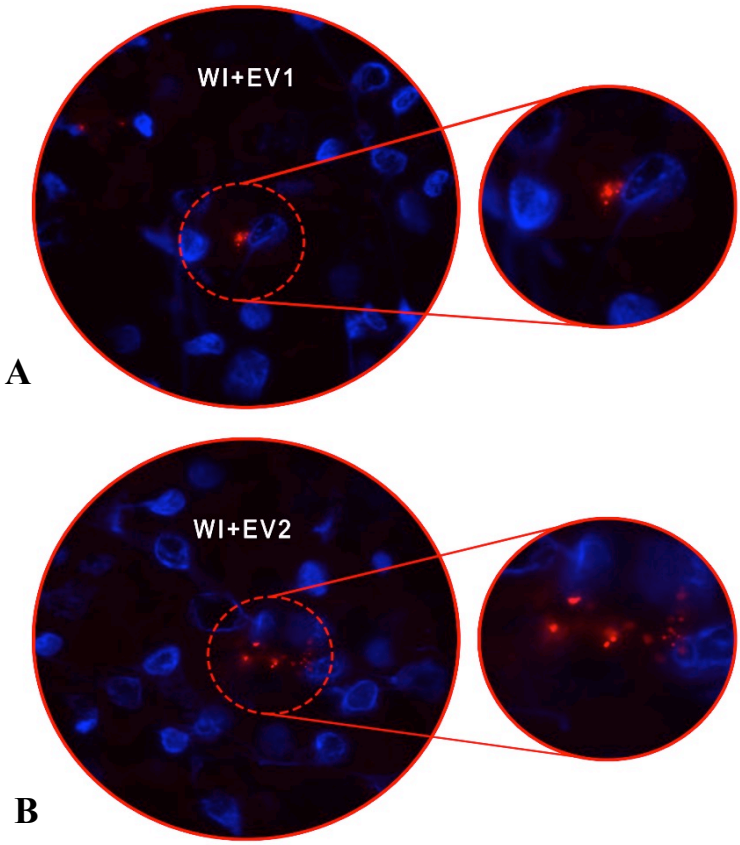


Figure 20. Epifluorescence microscopy images, in which liver parenchima cells can be seen in blue and HLSC-EV in red. [A] WI+EV1 group. [B] WI+EV2 group

Macroscopic aspect

The macroscopic appearance of the liver during NMP is an easily evaluable important indicator of the proper organ perfusion and of its quality. For example we took three pictures of each experiment at the beginning (t0), at the half (t3) and at the end (t6) of the perfusion time (figure 21). Qualitatively the difference is clear between NO INJURY livers, that appears well perfused and free of necrotic-ischemic sectors, compared to organs of other three groups in which the injury caused by the warm ischemia is evident, especially during the second part of the perfusion period. The external aspect evaluation, as expected, did not show any difference between treated organs and ischemic controls: the observation of macroscopic appearance is infact more useful to guide the regulation of perfusion parameters that to make judgments about treatment efficacy.

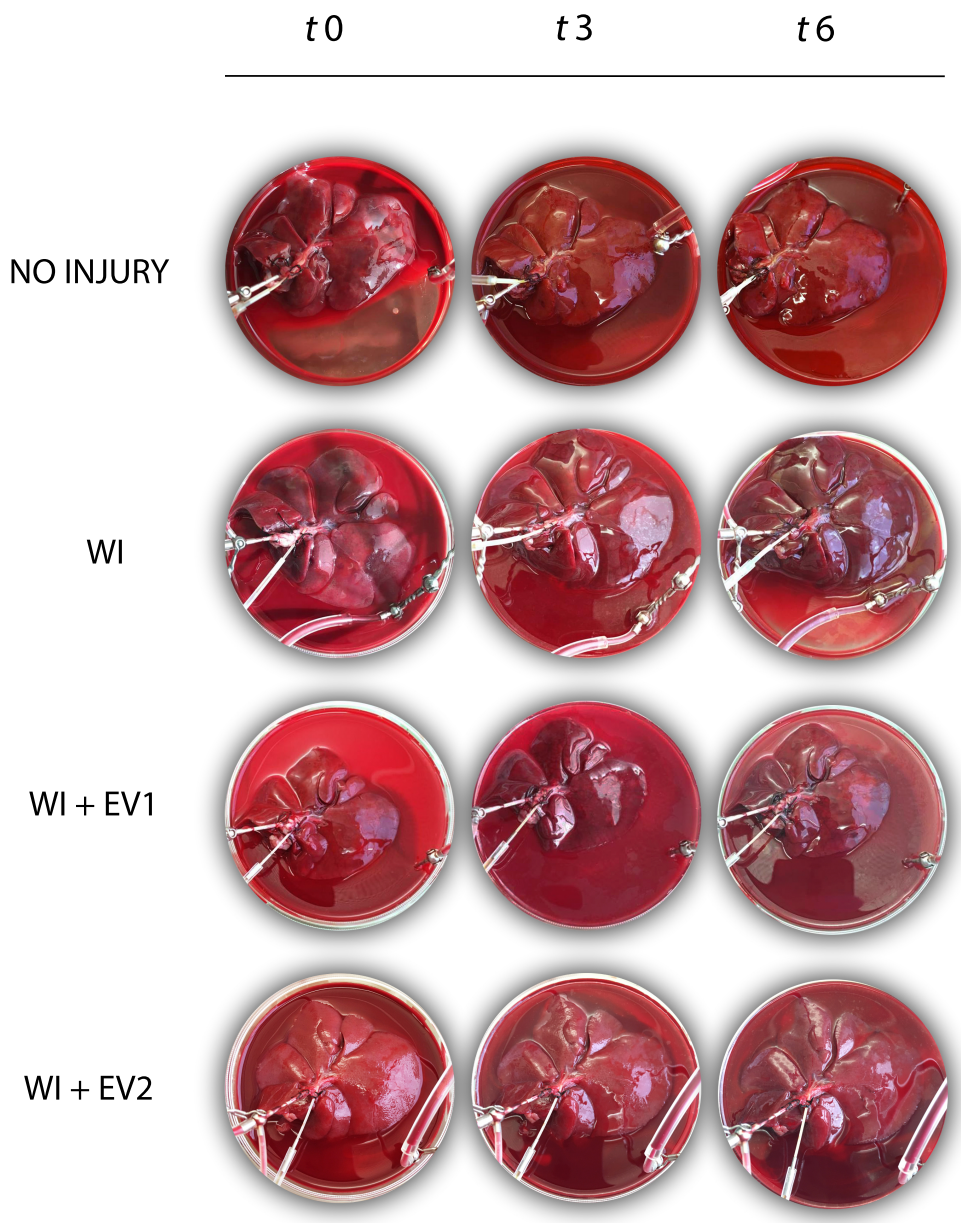


Figure 21. Pictures of lives perfused in NMP taken at timepoints t0, t3 e t6.

Hemodynamic parameters

Perfusion pressure was manually settled on values within 10 and 16 mmHg, on the basis of liver weight (table 10). Since the flow is regulated by PLUGSYS based on the selected pressure and the vascular resistances of the liver, the latter can be easily obtained by calculating the pressure/flow ratio. At the timepoint t6, corresponding at the end of the 6th hour of perfusion, NO INJURY livers showed resistance values significantly lower than WI and WI+EV1 groups ($p<0.0001$ and $p=0.038$,

respectively). In the WI+EV2 group, at t6, a relevant reduction of vascular resistance can be observed compared with WI group (p=0.016) (figure 22).

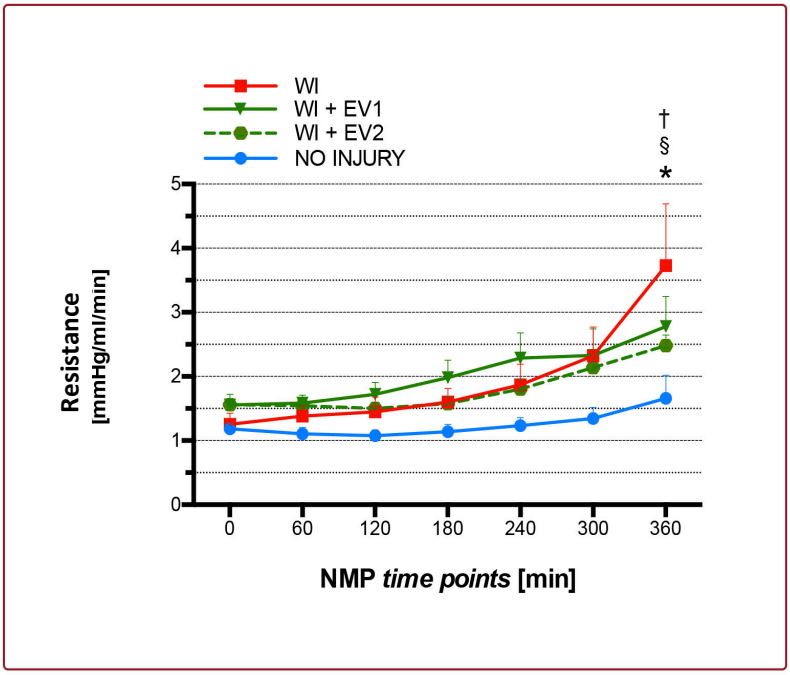


Figure 22. Resistance values during NMP. † = WI vs NO INJURY; § = NO INJURY vs WI+EV1; * = WI vs WI+EV2

Hepatic functionality during NMP

The study of liver function during perfusion was carried out by: 1) evaluating hourly bile production, 2) monitoring pH variations and the need of bicarbonate adding and 3) analysing perfusate samples taken every 60 minutes.

In almost all the livers of NO INJURY, WI+EV1 and WI+EV2 groups a continuous bile production during 6 hours of perfusion was observed, while in the WI group in 5 out of 6 cases, bile production stopped at the tird hour. At the timepoints t3, t4, t5 and t6 NO INJURY organs produced a quantity of bile significantly greater than WI and WI+EV1 organs (figure 23A). At the 6th hour (t6) the group treated with the higher dose ov EVs (WI+EV2) resulted to be able to produce anhigher quantity of bile compared to the controls (WI) (p=0.044) (figure 23A). Bile pH has been measured on collected samples every 60 minutes. Unfortunately the low bile volumes, expecially for the WI and WI+EV1 groups, did

not permit the measurement of pH by the probe in the majority of experiments (the probe need at least 150 µl of fluid volume). For this reason figure 23B curves are purely indicative and do not have any statistical value.

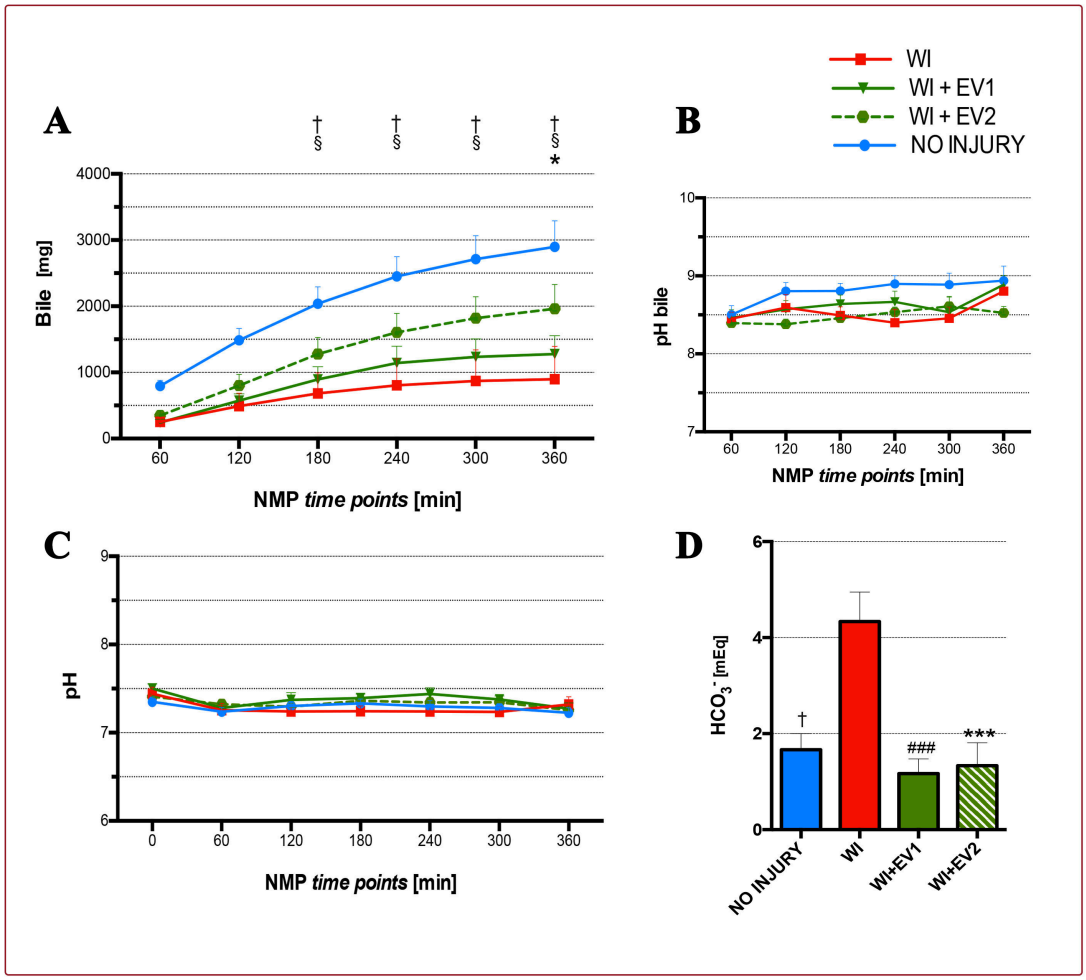


Figure 23. Metabolic parameters during perfusion. † = WI vs NO INJURY; § = NO INJURY vs WI+EV1; # = WI vs WI+EV1; * = WI vs WI+EV2. [A] Cumulative bile production. [B] Bile pH. [C] Perfusate pH. [D] Total bicarbonate mEq added during NMP.

In order to maintain perfusate pH over the minimum established value (≥7,30) we added aliquots of 1 mEq di HCO_3^-. Thanks to this support, in all the experiments a range of pH within 7,30 and 7,50 has been kept (table 10 and figure 23C). In the NO INJURY, WI+EV1 and WI+EV2 groups, livers were able to serf-adjust the perfusate pH almost autonomously; on the contrary in the WI group the acid-base balance resulted strongly compromised, with a tendency to acidosis, enough to need high bicarbonate quantities. The administration of HCO_3^- during perfusion resulted

significantly greater for WI group compared to the heathy controls of NO INJURY group (p=0.002), but also to treated organs with low dose of HLSC-EV (p=0.0004) and with high dose (p=0.0007)(figure 23D).

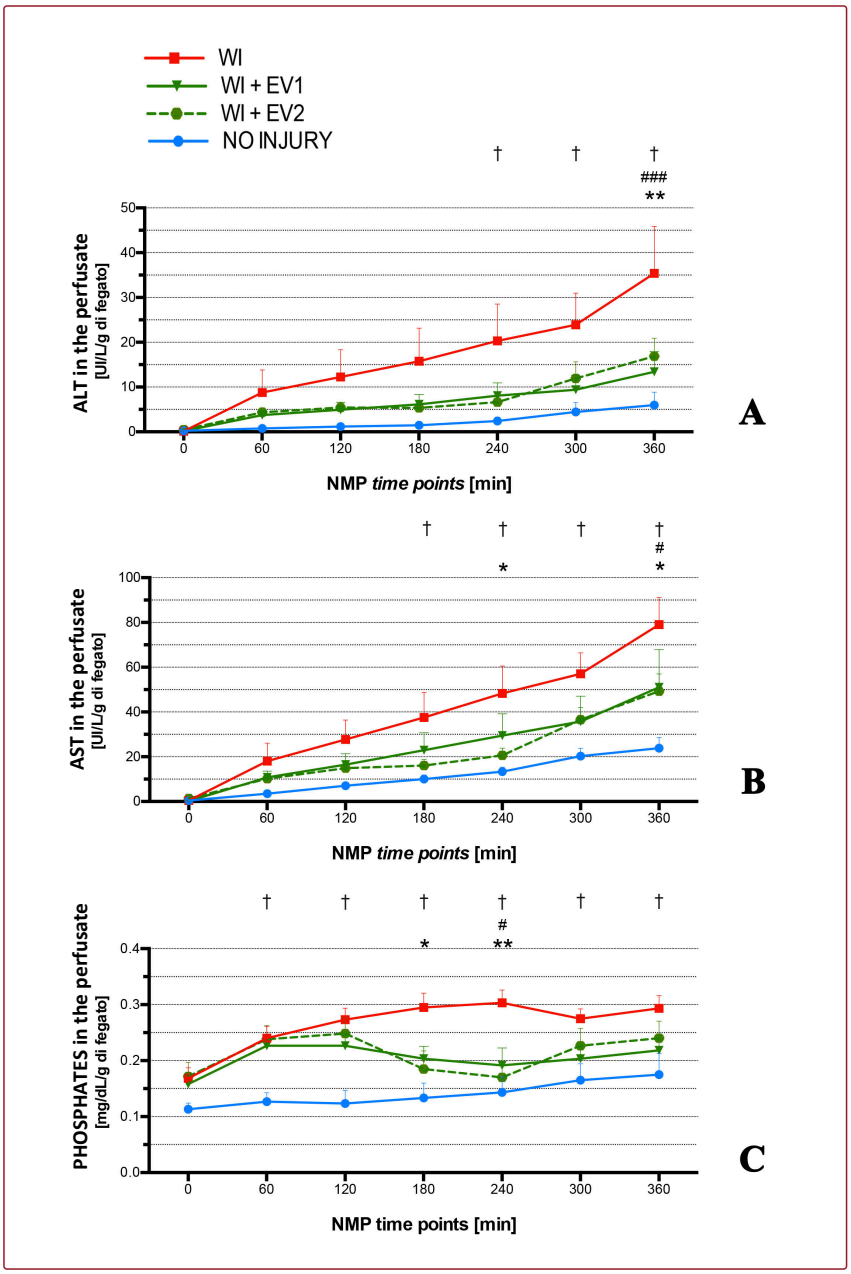


Figure 24. Transaminases and phosphate values measured in the perfusate. † = WI vs NO INJURY; # = WI vs WI+EV1; * = WI vs WI+EV2. [A] ALT. [B] AST. [C] Phosphate.

In all the experimental groups we observed a gradual and progressive increase of transaminases levels in the perfusate (figures 24A and B). The reached values were particularly high in the WI group, that compared to healthy controls of NO INJURY group released significantly higher quantities of ALT, from t4 moment

and AST from t3. Both the dosage of HLSC-EV were associated with a significant reduction of ALT at the end of perfusion (t6), with significantly lower levels compared with WI group, WI+EV1 group (p=0.0008), and WI+EV2 group (p=0.0069) (figure 24A). Concerning AST, also in this case both the doses of HLSC-EV allowed an important reduction of this enzyme compared to WI group at t6 (p=0.030 and p=0.018 respectively for WI+EV1 and WI+EV2); moreover the higher dose (WI+EV2 group) was associated with lower levels of AST compared to ischemic controls also at 4^{th} hours of perfusion (p=0.033)(figure 24B).

Phosphate values in the perfusate during NMP resulted significantly higher in WI group than NO INJURY group at every timepoint. In WI+EV1 group we observed lower phosphate levels than in WI group at t4 (p=0.010), while in the WI+EV2 group, the higher dose of HLSC-EV led to a significant reduction of phosphates compared to WI group, not only at t4 (p=0.001) but also at t3 (p=0.012)(figure 24C).

Histology and Immunohistochemistry

For histologic evaluation of biopsies taken at the end of perfusion, three stains were performed. H&E was used for a morphologic evaluation of the tissue, while for apoptosis and proliferative activity quantification immunohistochemistry stainings TUNEL and PCNA were performed.

The quantification of tissue injury has been measured with Suzuki's score. In almost all the livers, the hight score reached is due to the presence of moderate/severe edema (figures 25A, B, C and D). The score of WI group was significantly higher than NO INJURY group (p=0.021), and than WI+EV1 group (p=0.043)(figure 25E).

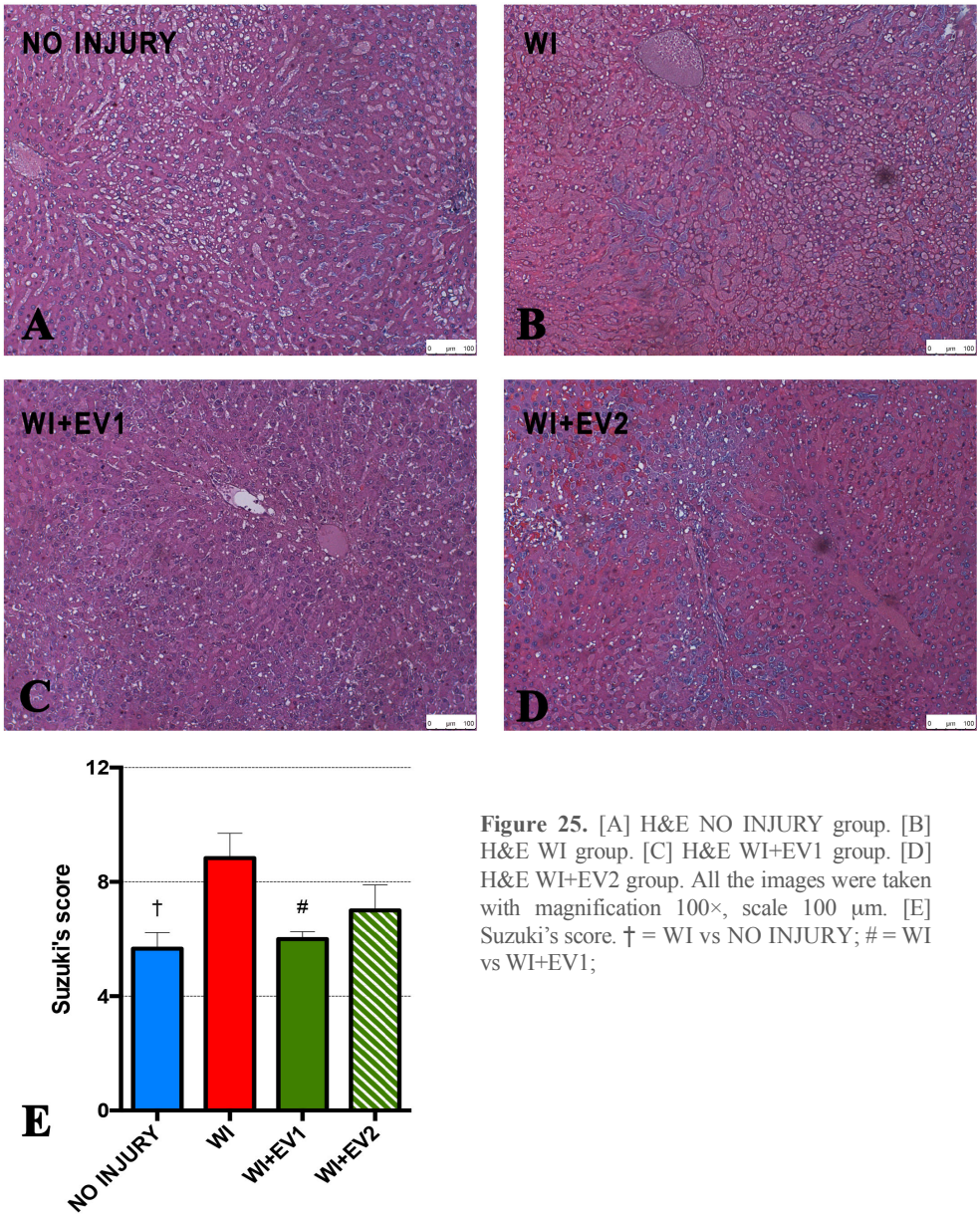


Figure 25. [A] H&E NO INJURY group. [B] H&E WI group. [C] H&E WI+EV1 group. [D] H&E WI+EV2 group. All the images were taken with magnification 100×, scale 100 µm. [E] Suzuki's score. † = WI vs NO INJURY; # = WI vs WI+EV1;

The count of mean number of TUNEL+ cells for field resulted in a low number of apoptotic cells in all the groups (mean of 5-10 cells for each liver), without any significant difference between groups, even with a trend towards an higher number of TUNEL+ cells in WI group (figure 26).

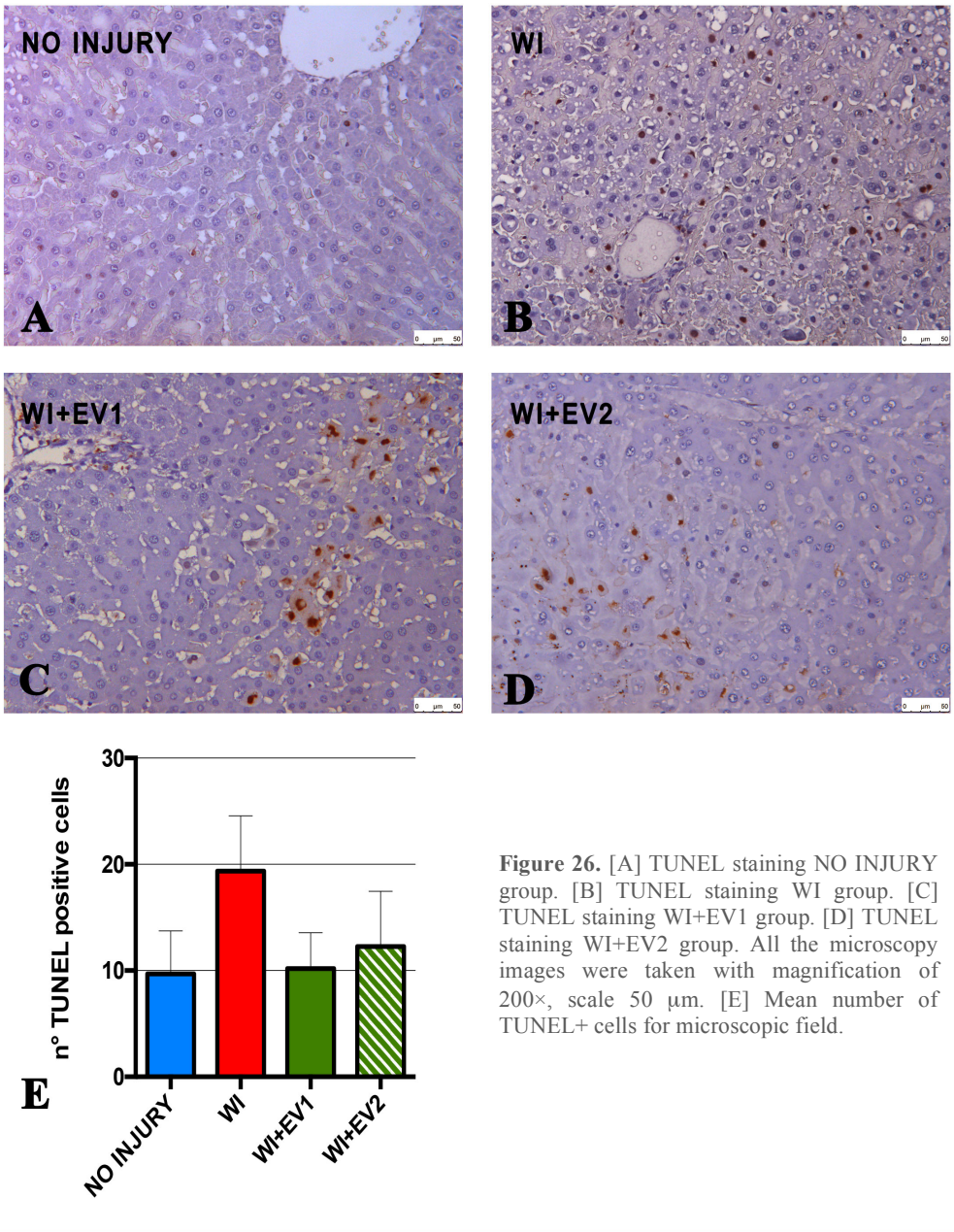


Figure 26. [A] TUNEL staining NO INJURY group. [B] TUNEL staining WI group. [C] TUNEL staining WI+EV1 group. [D] TUNEL staining WI+EV2 group. All the microscopy images were taken with magnification of 200×, scale 50 μm. [E] Mean number of TUNEL+ cells for microscopic field.

In tissues were PCNA staining was performed the proliferative index was expressed as the percentage of PCNA+ cells on the total number of cells in the slide. The percentage of proliferating cells in WI group was extremely low, with a mean slighly more than 1%, significantly inferior to NO INJURY group in which PCNA+ cells are 16% of the total on average (figure 27). In the treated groups the proliferative index was similar to NO INJURY group, but only in the WI+EV2 group a statistical significance was reached in comparison with WI (p=0.048)(figure 27E). On the other hand, the missed achieving of significance in the comparison between WI+EV1 and

WI proliferative index seems to be due to the marked variability of observed data in this analysis: it is possible to notice, infact, the wide standard error in figure 27E.

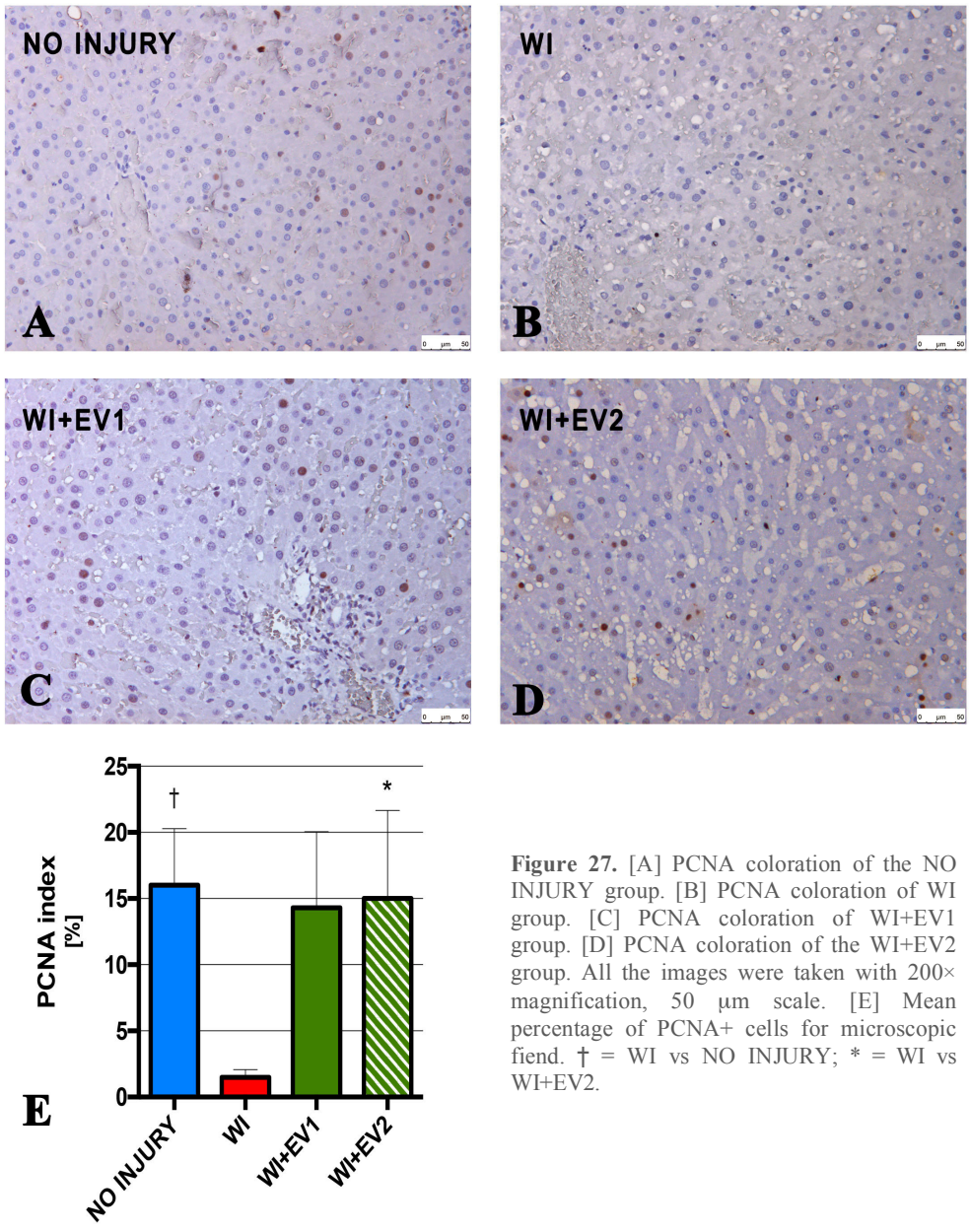


Figure 27. [A] PCNA coloration of the NO INJURY group. [B] PCNA coloration of WI group. [C] PCNA coloration of WI+EV1 group. [D] PCNA coloration of the WI+EV2 group. All the images were taken with 200× magnification, 50 µm scale. [E] Mean percentage of PCNA+ cells for microscopic fiend. † = WI vs NO INJURY; * = WI vs WI+EV2.

6.4 Discussion

The normothermic perfusion is an innovative technique of graft preservation with a proved effectiveness, expecially when applied on ECD livers.[77,78,80,107] Between the advantages of NMP there is the possibility to pharmacologically treat the livers during the preservation phase in order to further improve the quality of suboptimal grafts.[116,119] Considering this, NMP can be considered the most promising option for safely expanding the donor pool.

HLSC are stem cells resident into the hepatic parenchyma and provided of regenerative and hepatoprotective properties.[137,138] HLSC derived products, as the conditioned medium and the HLSC-EVs, are able to mimic the majority of HLSC effects, included mitogenic activity and apoptosis inhibition, by transferring proteins, mRNA and miRNA.[146,162]

Recently, in an hypoxic model of 4 hours of NMP, we demostrated the possibility to treat a liver graft with HLSC-EVs.[119] On the basis of these evidences, we decided to evaluate the efficacy of the HLSC-EVs tratment in a model of NMP similar to the clinical reality of ECD organs. In particular, we developed an experimental setting of DCD donation and increased the perfusion duration to 6 hours. After a first phase of NMP circuit optimization and standardization of the procedure, we set up a study including 4 experimental groups, one of these is representative of standard donation free of ischemic injury, the remaining three consist of organs exposed to 60 minutes of warm ischemia, in order to simulate a DCD donation condition. In two of the three DCD groups HLSC-EVs have been administrated at different doses to evaluate the effectiveness of this treatment for reducing IRI and to identify a possible dose-effect realation.

The standardization of experiments allows to obtain uniformity between study groups, that resulted statistically identical in terms of organs weight and operative parameters of NMP perfusion, leading to a valid statistical analysis of the research endpoints.

The internalization of HLSC-EVs into hepatic tissue is an integrin mediated mechanism (presumably led by α4-integrin),[146] and it has already been observed by our group in a precedent model of NMP, in which we found the presence of HLSC-

EV inside hepatocytes cytoplasm within 4 hours of perfusion.[119] In the present study the HLSC-EVs uptake in the liver has been further confirmed, as documented by epifluorescences microscopy images taken on tissues collected at the end of 6 hours of NMP (figure 20). Although in the groups treated with the higher dose of HLSC-EVs it seems to find a slightly higher quantity of EVs (figure 20B), this kind of microscopy analysis do not permit a quantitative statistical evaluation. The absence of an exact quantification of HLSC-EVs integration represents certainly a limit: the possibility to real time monitoring the presence of EVs in the perfusate or in the liver during NMP could allow a better understanding of their uptake dynamic, but unfortunately nowadays valid methods applicable for that purpose do not exist yet.

The observation of macroscopic aspect of the liver during perfusion represents one of the viability assessment parameters identified in NMP clinical studies NMP,[171] and it constitutes an evaluation of unquestionable immediacy and simplicity. In our study it was used to confirm the effectiveness of the experimental model of warm ischemia in causing an injury significantly highter compared to the normal livers (figure 21). Especially at the beginning of NMP, the evaluation of an homogeneous perfusion of the parenchyma allowed to modify the pressure and change the organ positioning in order to guarantee an adequate flow to every lobe.

Between viability assessment criteria identified by the group of Birmingham there is the stability of flow and pressure parameters.[171] In a controlled pressure NMP circuit, the resistance (calculated as the ratio between perfusion pressure and portal flow) represents a suggestive data of system hemodynamic. In all the groups we observed a progressive increasing of the resistances through time, particularly evident in groups subjected to ischemic damage (figure 22). The presence of a significantly higher resistance in WI e WI+EV1 groups compared with healthy controls during the last hour of perfusion, proves the detrimental effect of warm ischemia on hepatic microcirculation: it is well known that a prolonged WIT causes structural alterations of liver sinusoids that greatly compromise graft quality and its survival.[172] The higher dose of HLSC-EVs was able to significantly reduce the hepatic resistance compared to controls of WI group during the last 60 minutes of perfusion (figure 22). The ability of NMP to improve hemodynamic performance of graft has already been described[173] and it is known that intrahepatic vascular

resistances reduction is mostly dued to vasodilation mechanisms;[174] hence, the effect observed in the group WI+EV2, in addition to a decrease of the hepatocellular damage that certainly affect microcirculation functionality, could be related to a protective mechanism on sinusoidal setting. That hypothesis should be investigated with specific studies that analyze a possible cross-talk between HLSC-EV and hepatic endotelial cells.

Bile production and pH self-regulation ability represent two main indicators of metabolict activity of the liver during NMP.[107,175] Both in the NO INJURY group and in the HLSC-EVs treated groups bile production has almost always been maintained until the end of 6 hours perfusion, while in the WI group only in one case bile production continued over 3 hours, demostrating a marked metabolic suffering of the organs subjected to warm ischemia, perfused with NMP without the support of an additional treatment with EVs. In the WI+EV2 group the cumulative production of bile resulted significantly higher than controls of WI group (figure 23A). Bile acids are synthesized mainly by zone 1 and zone 2 hepatocytes, while HCO_3^- by zone 3 hepatocytes;[176] bile secretion depends also on cholangiocytes integrity. It follows that a good bile production during NMP is synonym of effective preservation of the whole parenchyma. HLSC-EVs administration at the higher dose resulted associated to a greater bile secretion, implying an effective protection of EVs for the mentioned cellular compartments. That data results particularly interesting because one of the major criticism in DCD transplantation is the rate of biliary complication.[43] Furthermore, the pH of bile produced during NMP is emerging as a parameter related with post-OLT ischemic cholangiopaty incidence;[175] unfortunately, the quantity of bile produced during our experiments (expecially in WI groups) was not enough to perform statistical analysis. The possible action of HLSC-EV treatment on improving the quality of bile, and then on limiting the biliary tree distress, could be an interesting point to investigate in larger size animal models. The significant reduction of HCO_3^- supplementation need in both the WI+EV1 and the WI+EV2 groups supports the hypothesis of an improved metabolism in liver treated with HLSC-EVs (figure 23D). The maintenance of acid-base balance by the liver depends strictly on its synthetic activity, in particular on glutammine metabolism and pH-dependent activity of glutaminases of zone 1.[177] Both the doses of HLSC-EV have

been able to promote the pH self-regulation in WI livers similarly to what happen for NO INJURY group.

Transaminases are a subclass of enzymes used in clinics as a surrogate marker of liver injury. In particular, in the specific setting of MP, ALT levels in the perfusate are considered a direct indicator of organ's viability,[178] while the post-OLT AST peak represents one of the main graft survival predictors and is commonly used as major outcome in NMP clinical studies.[110] In the present study NO INJURY group livers released low levels of transaminases in the perfusate, similarly to what described in litterature;[100] in WI group instead, we obrserved an AST and ALT curve very steep and significantly more elevated than the healthy controls (figures 24A and B), demostrating the inability of the NMP alone to efficiently protect DCD organs. On the contrary, both dosages of HLSC-EVs allowed a significative reduction of AST and ALT levels at 6th hour of perfusion compared to the ischemic controls (figure 24A and B), with a similar trend detectable for the whole lenght of perfusion but more evident after the 4th hour. Furthermore the most elevated dose of HLSC-EVs could anticipate this effect compared to the lower dose, as confirmed by the presence of AST values already significantly lower at t4 in the group WI+EV2 (figure 24B). These data agree with what observed in our previous hypoxic NMP model,[119] and demostrate again the ability of HLSC-EVs to protect parenchima integrity and reduce hepatocyte cytolysis during NMP.

Serum phosphate levels are a clinical parameter that correlates with prognosis of patients affected by fulminant hepatitis and can be used as marker of metabolic function of the liver.[179] In particular, phosphate is a fundamental substrate for mitochondrial ATP synthesis, hence an increase of its concentration is indicative of aerobic respiration reduction and an energetic deficit of the cell. ATP depletion represents one of the critical point in IRI damage for DCD organs, indeed, in WI group livers phosphate levels were significantly higher than controls for the whole duration of perfusion (figure 24C). In the WI+EV2 group we observed a clear inversion of phosphate trend between the tird and the fourth hour of perfusion, leading to the assumption of a metabolic activation and a restoration of ATP synthesis mediated by the treatment (figure 24C); in the WI+EV1 group the appearance of the curve is very similar to WI+EV2 group, but the significativity

compared to WI controls has been reached only at the fourth hour of NMP, confirming again a better efficacy of the higher dose of HLSC-EV especially for temporally anticipation of the treatment effects (figure 24C). The second detectable change of slope of the curve between t4 and t5 in both the treated groups could signify a loss of protective effects of HLSC-EV. Based on this observation it would be interesting to consider the possibility of administration of a second dose in itinere (for example at t3), to prove a possible extension of the interaction between HLSC-EV and the metabolic activity of the liver also during the second part of perfusion.

Histological analysis have been performed both on tissues labeled with H&E and with immunohistochemistry staining. In particular, the morphologic evaluation has been carried out using a specific score for IRI damage;[170] it was observed a strong presence of interstitial edema in all the tissues (figures 25A, B, C and D), probably attributable to the hight pressure levels to which the portal circle has been subjected (table 10) and to the possible presence of an obstacle to venous flow for the missed cannulation of VC. Premised this confounding factor, the score attributed to WI organs was significantly higher than healthy controls, as predictable, but also than WI+EV1 livers (figure 25E), evidencing a protective effect on tissue necrosis HLSC-EVs mediated, as already observed in previous studies.[119] The failure to achieve a statistical significance in the comparison between WI and WI+EV2, despite a very similar trend to the group WI+EV1, could be caused by an anticipation of the effect of the higher dose (as seen in biochemical results) with consequent loss of the differences at the moment of bioptic sample (t6). Similarly the absence of results in TUNEL analysis would suggest an incongruence between the HLSC-EVs anti-apoptotic effect already observed in previous studies after four hours of NMP,[119] and the timing of histological observation (6^{th} hours). For that purpose, it would be very interesting to evaluate the cytokines profile and the expression of apoptotic markers at every perfusion timepoint. The study of the proliferative activity performed with PCNA staining showed the presence of actively proliferating cells both in the NO INJURY group and in WI+EV1 and WI+EV2 groups, with a percentage near to 15% of the total (figure 27E). On the contrary, in untreated ischemic livers (WI group), the cellular proliferation was almost absent and significantly lower than the healty controls and the group treated with the higher dose of HLSC-EV (figure 27E). On

the other hand, the absence of differences between the WI group and WI-EV1 group seems to be caused by the excessive intra-group variability of data, that proves that the proliferative stimulation iduced by vescicles wes present in some experiments but not in others. On the whole, PCNA results confirm the ability of HLSC-EVs to vehiculate nRNA promoting tissue regeneration inside liver parenchima;[146] that observation makes the treatment with HLSC-EV very promising for ECD organs reconditioning in long duration perfusions.

In conclusion, in the present study we developed an experimental perfusion model of DCD livers with NMP. The adequacy of the warm ischemia model as a surrogate of DCD donation is testified by the great difference between ischemic organs quality compared to the controls in terms of hemodynamic, metabolic biochemical and histologic parameters. In this setting, we used two different doses of HLSC-EVs to evaluate the protective efficacy on DCD livers. Both the doses were able to reduce the release of cytolysis enzymes during NMP and to promote cellular metabolism, stimulating bile production, phosphate consumption and pH self-regulation. Moreover, the hightest dose of HLSC-EV had a positive effect on vascular resistance and cellular proliferation.

Even though these promising results it is mandatory to highlight some significant limits of our study. First of all the sample size is still very small and we hope in the future to collect an higher number of cases both to describe the IRI process and to strongly demostrate our theories. The other important limitation is the difficulties in translating our small animal model in an human setting. First of all our experiment does not consist in a real transplantation model that can demostrate the correlation between the positive effects of HLSC-EV on the parameters evaluated in course of NMP and an actual improvement of post-OLT outcomes. It follows the necessity of developing in our lab a model of isolated whole blood perfusion or a model of murine liver transplantation, to demostrate the improved function of organs reconditioned with HLSC-EV also after “in vivo” reperfusion. If our hypothesis were confirmed, the efficacy of NMP reconditioning will be tested than in other experimental models more similar to clinical practice (for example a porcine model of liver transplantation with hight risk donation) and subsequently in a safety and feasibility human trial.

The clinical application of this technique could lead to an increased number of liver accepted for transplantation reducing waiting list time and mortality, to an improvement of public offer and a decrease of debilitating complications of terminal liver diseases and costs related to them.

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