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Machine Perfusion and its Impact on Liver Transplantation

Ph.D. Thesis

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List of Abbreviations

ECD	Extended-criteria donor
PNF	Primary non-function
EAD	Early allograft dysfunction
ATP	Adenosine triphosphate
MP	Machine perfusion
CS	Cold storage
HA	Hepatic artery
PV	Portal vein
IVC	Inferior vena cava
SCS	Static cold storage
CI	Cold ischemia
IRI	Ischemia-reperfusion injury
HMP	Hypothermic machine perfusion
ROS	Reactive oxygen species
NMP	Normothermic machine perfusion
SNMP	Subnormothermic machine perfusion
NRP	Normothermic regional perfusion
AST	Aspartate transaminase
ALT	Alanine transaminase
AP	Alkaline phosphatase
GGT	Gamma-glutamyl transferase
HOPE	Hypothermic oxygenated perfusion
UW	University of Wisconsin
ROS	Reactive oxygen species
HES	Hydroxyl-ethyl starch
RBC	Red blood cell
HTK	Histidine-tryptophan-ketoglutarate
PEG	Polyethylene glycol
IGL-1	Institut George Lopes
LDH	Lactate dehydrogenase

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CIT	Cold ischemia time
RBC	Red blood cell
HBOC	Hemoglobin-based oxygen carrier
ITBS	Intrahepatic bile duct strictures

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1. Introduction and Background

1.1 Introduction

Liver transplantation has been established as the only treatment for end-stage liver diseases. Since it was first performed in the 1960s and thanks to significant breakthroughs in the development of immunosuppressive drugs, surgical and anesthetic methods, in-depth understanding of the liver's pathophysiology and the changes that occur in the case of disease it has emerged to be the standard treatment for liver failure, exhibiting high success rates. Of note, the synergy of several scientific fields such as medicine, engineering, biology and chemistry has been of great importance in overcoming many challenges and proving liver transplantation to be an effective surgical treatment.

The success of liver transplantation as a standard procedure, in combination with the ageing population and the rising incidence of liver diseases worldwide, has led to an increase of the number of patients in need of a liver transplant over the last decades. On the other hand, the number of available liver grafts does not meet the growing demand. As a result, a shortage of donor organs occurs, leading to an increase in the waiting list mortality, as the demand for donor livers remains unmet in several cases. In 2021, a total of 2411 registrations were made in the Eurotransplant liver waiting list (1) and 407 deaths on the waiting list were recorded (2). Notably, the probability of death in the EU waiting list 3 years after registration reaches 20%, based on registrations in the period 2011-2015 (3).

In an attempt to reduce the discrepancy between the demand and the availability of donor livers, transplant centers have extended the criteria for acceptance of marginal livers (4). The utilization of such extended criteria donor (ECD) grafts includes donation after circulatory death (DCD), steatotic livers and organs from donors of advanced age (4-9). However, these types of grafts bear high risk of post-transplant complications such as primary non-function (PNF), early allograft dysfunction (EAD), biliary complications and even graft loss (10-13), with consecutively needed re-transplant. Consequently, novel approaches are emerging, aiming to assess the functionality of the liver, improve the preservation quality and, if possible, repair the organ prior to transplantation; thus, clinical outcome can be predicted and ultimately improved (14, 15). Therefore, the utilization of ECD grafts strongly relies on optimizing the preservation conditions. Machine perfusion (MP), a dynamic preservation technology, has emerged to safely expand the donor pool

by extending the preservation period, improving the functionality and quality of the graft, and allowing real-time monitoring of the graft's viability or resuscitation marginal livers (16-19).

In principle, machine perfusion is a dynamic ex-situ preservation method. A perfusion fluid is circulating through the organ, providing the cells with nutrients and oxygen, and flushing metabolic products. However, the concept of machine perfusion is not novel. The first liver perfusion machine was developed by Alexis Carrel and Charles Lindbergh in 1935 (20). It was completely made out of glass, capable of performing ex-vivo perfusion of an entire organ and was successfully used for ex-vivo preservation of animal livers (21). Machine perfusion was applied to human liver transplantation for the first time in 1967 by Thomas Starzl, who utilized a peristaltic DeBakey pump in order to perfuse the liver with cold, oxygenated blood through the portal vein (22). Despite the promising results, further research and the concept of machine perfusion were abandoned due to the high costs and complexity compared to simple cold storage (CS). The development of several cold storage solutions led to remarkable preservation outcomes (23). Thus, the efficiency and safety in standard-quality grafts, cost effectiveness and logistic simplicity of CS, have established it as the gold standard for organ preservation until today (7, 15). Nevertheless, the inability of CS to optimally preserve low-quality ECD grafts and the increased utilization of marginal grafts in order to expand the donor pool, has now renewed scientific interest and research in machine perfusion (4, 24, 25).

The temperature setting is of substantial importance in machine perfusion and it can be determined over a wide range. The selected temperature dictates the level of cellular metabolism for ATP production as well as the degree of oxygen demand (26). Although the limits are not strictly defined, machine perfusion is classified into temperature sections: hypothermic (0-12^{o}C), subnormothermic (20-34^{o}C) and normothermic (35-38^{o}C) (8, 27). The metabolic requirements of the liver increase, alongside with the temperature at which machine perfusion is applied. Thus, different temperatures affect the organ in a different manner and tolerate different timing strategies in the duration of perfusion prior to transplantation (15, 28).

Generally, machine perfusion devices are technologically demanding and complex configurations. Apart from the temperature, which is the main independent parameter for their operation, there are several adjustable settings as well, which are interconnected and strongly affect the graft and preservation outcome. Since different temperatures affect the organ in a different manner, they require different timing

strategies and duration of perfusion as well as different operating parameters such as oxygenation, perfusion route, mode of flow and pressure, and perfusion solutions (8). Therefore, optimizing the preservation outcome is a multifactorial problem that requires good understanding of the anatomy and metabolic needs of the liver as well as the technical modalities of the device.

Although machine perfusion techniques are well-defined based on temperature, the selection of suitable perfusion settings for each technique has been a matter of debate throughout the history of the field. Namely, the lack of consensus regarding the selection of optimal perfusion parameters such as perfusate, type of flow (continuous vs pulsatile) and perfusion circuit (single vs dual) led to the development of several experimental setups and numerous combinations of perfusion settings over the last years (29-31). Nowadays, there are clear indications for the selection of certain perfusion parameters, such as oxygenated perfusate and dual perfusion. However, the interplay of these parameters and their effect on the preservation outcome, when applied in each machine perfusion technique, still remains partly unclear. In addition, each technique offers its own unique advantages and is suitable for different perfusion duration, but there is uncertainty on which approach is the best for effectively achieving long-term perfusion.

1.2 Liver Anatomy, Physiology and Pathology

1.2.1 Anatomy

Understanding the major anatomical characteristics of the liver is of great importance for the development of liver MP systems. Particular emphasis is given on the microscopic anatomy of hepatocytes and cholangiocytes, as they play a crucial role in the major functions of the liver. In addition, an overview of the liver blood supply is given, as it provides the basis for perfusate circulation during dynamic preservation. MP configurations aim at matching liver anatomy and emulating a physiological environment in order to effectively preserve it, thus knowledge of the major anatomical and physiological characteristics as well as the metabolic needs of the liver is essential.

1.2.2 Gross Anatomy

The liver is the largest internal organ of the body, weighing approximately 1.4 kg. It is located in the upper and right parts of the abdominal cavity, where it lies under the diaphragm and almost completely enclosed by the thoracic cage (32).

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Externally, the liver has four primary lobes. The right lobe, which is the largest, is separated anteriorly from the left lobe by the line of attachment of the falciform ligament. The gallbladder lies on a recess in the inferior part of the right lobe. The other two lobes, the posteriormost caudate lobe and the quadrate lobe, which is situated inferior to the left lobe, are visible in an inferior view of the liver, as depicted in **Figure 1** (32, 33).

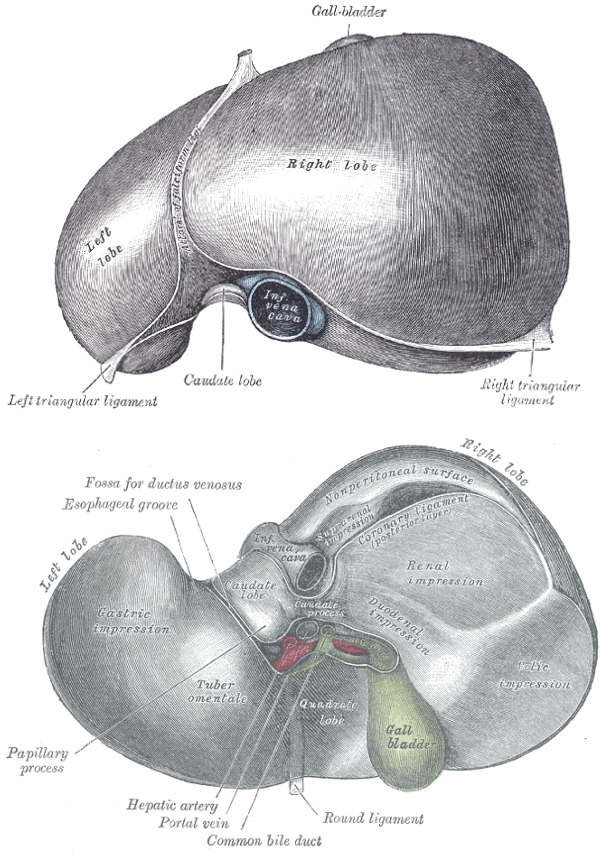

Figure 1. Superior (left) and inferior (right) view of the liver (32).

Of note, the lobe scheme is solely based on superficial and external features of the liver, thus is typically not adopted during hepatic surgery. Instead, the Couniaud classification system is used, which divides the liver into eight functinally independent segments. Each segment is characterized by its own vascular and biliary supply (34, 35). This system allows removal of liver sections during surgery, while avoiding most of the major vascular structures and inducing the lowest possible risk in the patient (33).

1.2.3 Microscopic Anatomy

In the microscopic level, the structural and functional units that constitute the liver are the liver lobules (**Figure 2**). Each lobule is characterized by a hexagonal shape and consists of hepatocytes (33). A central vein passes along the longitudinal axis of each

lobule. The hepatocytes radiate outward from the central vein, towards the periphery of the lobule. Each of the six corners of the lobule contains the portal triad, a distinctive feature of the lobule, which is composed a branch of the hepatic artery and portal vein, a bile duct as well as lymphatic vessels and a branch of the vagus nerve (33, 36). In the space between hepatocytes, there are the liver sinusoids, which are enlarged and heavily fenestrated capillaries (36). Blood from the hepatic artery and portal vein is drained from the triads, filtered through the sinusoids and empties into the central vein. Then, blood enters the hepatic veins, which convey it from the liver and empty into the inferior vena cava.

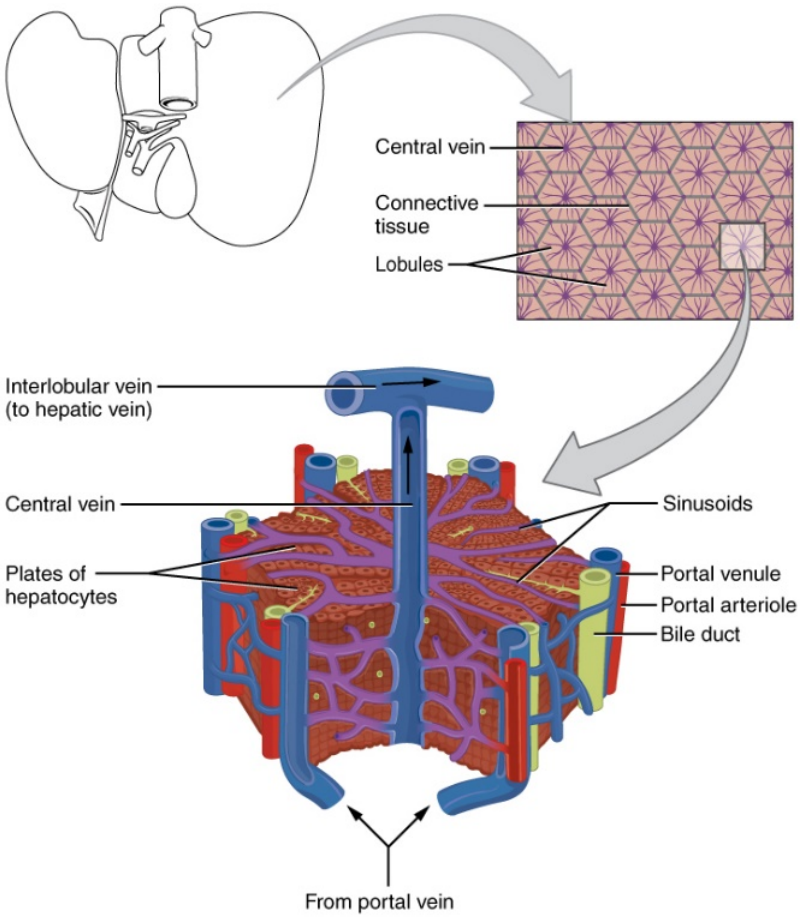

Figure 2. Microscopic anatomy of the liver (33).

Hepatocytes are versatile cells and account for approximately 75-80% of the liver volume. They contain large amounts of both rough and smooth endoplasmatic reticulum (ER), peroxisomes and mitochondria (33). Hepatocytes utilise oxygen to synthesize ATP via cellular respiration, which allows them to perform a wide range of metabolic and secretory functions, including bile synthesis.

However, bile secretion in the liver is a process that occurs in two stages (37) and involves the participation of hepatocytes as well as cholangiocytes, epithelial cells that line the intrahepatic bile ducts (38). Initially, hepatocytes secrete primary (canalicular) bile, which flows through the bile canaliculi of the lobules towards the bile duct branches of portal triads (33, 38). Then, as canalicular bile flows through the bile ducts, it is modified by cholangiocytes through a series of secretory and reabsorptive processes, which involve the transport of water as well as various ions and solutes between cholangiocytes and canalicular bile (38, 39). Bile modification is an intricate, hormone-regulated process and eventually leads to the dilution and alkanization bile (40), as it flows through the biliary tree to be delivered to the gallbladder or the duodenum. Of note, the secretion and absorption mechanisms of cholangiocytes are regulated mainly by hormones, peptides and nucleotides through several intracellular signaling pathways (38, 39), which require release of ATP (41). Therefore, it is clear that oxygen uptake is necessary for hepatocytes and cholangiocytes, as ATP plays a crucial role in their respective physiological functions and especially in bile synthesis.

1.2.4 Functional Anatomy

The secreted bile flows through the bile ducts of the liver biliary tree, which ultimately fuse into the common hepatic bile duct. Along its course toward the duodenum of the small intestine, it merges with the cystic duct which drains the gallbladder to form the bile duct (33). The secreted bile is either carried to the gallbladder by the cystic duct or directly to the duodenum by the common hepatic duct.

The blood vessels connected with the liver are the hepatic artery (HA), portal vein (PV) and the hepatic veins, which convey blood from the liver and empty in the inferior vena cava (IVC). The central area of the liver - known as hepatic hilum -, contains the opening called porta hepatis, which is the point of entrance for the HA and PV (33, 36). The IVC is located in a short depression, called fossa for the inferior vena cava, which extends obliquely upwards on the posterior surface next to the caudate lobe (32).

The liver receives approximately 25% of the cardiac output and its blood supply comes from the HA and PV. Hepatic blood volume ranges from 25-30 mL/100g liver weight, corresponding to 10-15% of the total blood volume (42). PV delivers 75-80% of the overall liver blood supply; portal venous blood is drained from the spleen, stomach, small and large intestine, gallbladder and pancreas (43). This blood is rich in nutrients and partially deficient in oxygen. The remaining 25% is oxygen-rich blood, supplied by

the HA (44). PV is a low-pressure/high-flow circuit, whereas HA is a high-pressure/low-flow system. Each of these blood supply circuits account for approximately 50% of the liver oxygen demands (42, 44). Mean HA pressure is comparable to the aortic pressure, while PV pressure typically ranges between 6-10 mmHg (42, 45).

Both afferent blood supply branches merge at the sinusoidal bed of the liver (42). Subsequently, blood flows through the sinusoids and eventually empties into the central vein of each lobule (32). As mentioned, the central veins coalesce into hepatic veins, which drain the liver and empty into the IVC; in turn, IVC conveys the blood from the liver to the heart. It should be noted that these three major blood vessels -PV, HA and IVC- are targeted in MP systems. Their physiological characteristics are typically emulated by the device's elements to accomplish successful perfusion of the organ.

1.3 Functions

The liver is serves a wide range of both metabolic and regulatory functions in the body, the most important of which, include (46, 47):

- Bile synthesis and secretion
- Bilirubin absorption and metabolism
- Storage and metabolism of glycogen, minerals and vitamins
- Metabolism of fat, proteins and carbohydrates
- Regulation of blood clotting via albumin and clotting factors synthesis
- Drug metabolism and blood detoxification; removal of toxic substances such as alcohol and other drugs
- Prevention of infections by synthesizing immune factors and removing bacteria from blood

1.4 Diseases of the liver and liver transplants

1.4.1 Liver diseases and causes of transplantation

Liver transplantation becomes necessary due to a permanent inability of the liver to function. In children, bile duct malformations are usually the reason for transplantation, in adolescents mostly metabolic diseases and in adults terminal cirrhosis(48) A variety of diseases can be the cause of functional incapacity, including liver cirrhosis caused by: (49)

- Alcoholic cirrhosis caused by chronic alcohol abuse
- Hepatitis B
- Combined hepatitis B and hepatitis D
- Hepatitis C
- Autoimmune hepatitis
- Primary biliary cholangitis (PBC)
- Primary sclerosing cholangitis (PSC)
- Metabolic diseases
- Polycystic degeneration
- Familial amyloid polyneuropathy
- Budd-Chiari syndrome
- Caroli syndrome
- Liver cancer
- Fulminant liver failure (examples: Tuberous leaf fungus poisoning, acetaminophen poisoning, fulminant viral hepatitis)
- Neonatal hemochromatosis

1.4.2 Pathogenesis of liver cirrhosis

Liver cirrhosis, the most common cause of liver failure in adults, is usually caused by alcoholism, fatty liver and viral diseases (especially hepatitis C). Alcoholism is the most common cause of liver cirrhosis in industrialized countries, accounting for about 50% of cases. Massive alcohol consumption and the resulting high metabolization rate of ethanol to ethanal lead to a sharp increase in the nicotinamide adenine dinucleotide quotient in the body. Fatty liver represents the most common liver finding in Germany. This liver disease is characterized by fat storage in the liver cells, which is still reversible in the initial course, but often leads to cirrhosis in the later stages. Especially in untreated HCV infection, up to 20% of all cases develop cirrhosis, but often only after 20 to 30 years.

The cause of cirrhosis is necrosis (death) of liver cells, caused, for example, by viruses or toxins. The cells release cytokines that activate liver macrophages (Kupffer cells) and fat storage cells of the liver (Ito cells) on the one hand and monocytes and granulocytes from the blood on the other. These cells cause destructive remodeling of the organ structure with parenchymal necrosis, formation of regenerative nodules (pseudolobules) and connective tissue septa. These connective tissue nodules severely disrupt the vascular

structure underlying all liver functions: All channels of the liver, comprising those that bring bile to the gallbladder via the bile ducts (canaliculi and ductus) as well as the vessels that carry nutrients from the portal blood to the body, flush pollutants to the hepatocytes for detoxification, and supply the liver with oxygenated blood are affected. Bile ducts may reform but end up blind. As a result, blood congestion occurs between the liver and the digestive tract (portal hypertension), causing ascites to form and the spleen to enlarge. In the worst case, esophageal variceal bleeding occurs. Hepatocyte failure also causes hepatic encephalopathy: in liver cirrhosis, ammonia metabolism is reduced by up to 80%, with ammonia formed in the intestine bypassing the liver via vascular collaterals. The lack of degradation increases toxin concentrations in the blood and ammonia passes the blood-brain barrier. Astrocytes in the brain swell and cerebral edema may develop, leading to cognitive deficits that are usually episodic. Ultimately, the inadequate detoxification function of a cirrhotic liver can lead to hepatic coma. In this context, hepatic encephalopathy is considered a predictor of a particularly severe course of liver cirrhosis. In one study, for example, nearly half of all liver cirrhosis patients with hepatic encephalopathy died within one month of diagnosis. Study data also show a reduction in risk for other liver cirrhosis complications such as spontaneous bacterial peritonitis (SBP) or variceal hemorrhage when hepatic encephalopathy is treated. The pathologist differentiates between patients with hepatic encephalopathy and those with hepatic encephalopathy(50).

Pathologists differentiate between micronodular, macronodular, and mixed nodular cirrhosis based on the external appearance of the organ. The liver shrinks and its surface becomes wrinkled and nodular. Microscopically, active or florid (i.e., advancing) and inactive cirrhosis can be distinguished. The precursor of liver cirrhosis is liver fibrosis(51).

1.4.3 Management of liver transplantation

After a suitable donor organ is found for the person waiting, time is the most important factor. The organ should be transplanted into the recipient's body ideally within 8 hours after harvesting, as its functionality deteriorates rapidly. While 12 hours often are recommended as a cut off for the cold ischemia time, there are reports of successful transplantation after longer ischemia times. During this time, the organ is checked for transplant viability and taken to the organ recipient's transplant center. This time window is further shortened by the duration of the actual surgery, which often exceeds 8 hours.

Unlike other organs such as kidney or pancreas, liver transplantation is an orthotopic transplantation. This means that the new organ is implanted in the same place in the

body as the previously removed old one (abbreviation OLT = Orthotopic Liver Transplant). This more complicated procedure is necessary because the vascular supply of the liver with its three vessels (inferior vena cava, portal vein, hepatic artery) and the bile duct can only be guaranteed at this location in the body (52-55).

Through a large upper abdominal incision, the liver is mobilized, and blood vessels and the common bile duct are dissected. Cirrhotic disease often causes portal hypertension, which, like the coagulation disorders that often occur in liver disease, can have a complicating effect on the explantation of the old liver (52-55).

While the liver is still being removed, the donor liver is prepared for implantation. The four central steps after insertion of the new organ are the connection of the suprahepatic inferior vena cava of the liver graft with the vena cava of the recipient. This can be done in piggyback or cava replacement technique. The latter requires an additional infrahepatic caval anastomose. Then the reconstruction of the portal vein takes place. After this anastomosis, the portal vein is opened and porto-venous re-perfusion of the liver takes place. Depending on the used preservation solution there are different ways to remove them in order to avoid metabolites from cold storage from circulating in the recipient.

This is followed by the hepatic artery reconstruction and consecutive arterial reperfusion of the liver. The biliary reconstruction is the last step and less time critical. It can be performed as duct-to-duct or bilio-enteric reconstruction by using a Roux limb of the small bowel(52-55).

1.4.4 Risks and Complications

In addition to the complications possible with any operation, such as wound infections, pneumonia, the possibility of thrombosis with subsequent pulmonary embolism, among others, liver transplantation also harbors some typical complication possibilities. The main early complications after liver transplantation consist of primary non-function of the transplant liver, which may require emergency re-transplantation. There is an increased risk of bleeding due to the sometimes-poor blood clotting caused by liver disease and the need for vascular sutures. In 1-2% of transplants, occlusion (thrombosis) of the hepatic artery occurs, often necessitating repeat liver transplantation in the long term. Leakage or narrowing (stenosis) may occur at the bile duct reconstruction. The risk of infection, especially with regard to fungal and viral infections, is increased due to the necessary immunosuppression (56-58).

In the event of a rejection, it is extremely important to be aware of the warning signs. Possible clinical signs of a rejection are: Feeling of weakness, rapid fatigue, elevated temperature above 37.5° C for several hours, loss of appetite, pain in the abdomen, clay-colored stools, dark urine, and yellowing of the eyes and skin. Nevertheless, the diagnosis of acute rejection is made on the basis of laboratory (hourly / daily monitoring of liver function parameters) and histological criteria (needle biopsy). The therapy of acute rejection consists in the application of high-dose cortisone (methylprednisolone (500mg) for three consecutive days. At the same time, baseline immunosuppression is increased, and an additional immunosuppressive drug is added if necessary. In the presence of cortisone-resistant rejection, therapy is followed with antibody therapy directed against T cells for 3 to a maximum of 10 days. Due to the improvement in immunosuppression, chronic rejection is rarely observed after liver transplantation (56-58).

Late complications after liver transplantation include recurrence of underlying disease (hepatitis, liver tumor) and chronic liver failure with the need for liver re-transplantation. The general tumor risk is increased to about 3-fold due to immunosuppression. Furthermore, the necessary anti-rejection drugs can lead to diabetes mellitus, hypertension, and slow kidney failure with the ultimate need for dialysis (59, 60).

1.4.5 Epidemiology of liver cirrhosis and transplantation

The incidence of liver cirrhosis in Europe and the USA amounts to approximately 250 cases per 100,000 people every year. An increase in the incidence of liver cirrhosis is expected in the coming years. Cirrhosis is characterized by poor life expectancy and is considered one of the leading causes of morbidity and mortality: in the United States, it is the 12th leading cause of death (9.5/100.000 persons). Even higher numbers are thought to occur in Asia and Africa. Men are affected twice as often as women (61). It is possible that the prevalence is underestimated and underreported, as a population-based study from the United States with a total of 633,323 adults indicated an overall prevalence of 0.27% (62). Cirrhosis is a leading cause of mortality and morbidity across the world. It is the 11th leading cause of death and 15th leading cause of morbidity, accounting for 2.2% of deaths and 1.5% of disability-adjusted life years worldwide in 2016. Chronic liver disease caused 1.32 million deaths in 2017, approximately two-thirds among men and one-third among women (63, 64).

It is estimated that there are approximately 300,000 - 400,000 patients with liver cirrhosis living in Germany, of whom approximately 20,000 die each year as a result of the disease (65). The most frequent causes of liver cirrhosis in Germany are alcoholic and non-alcoholic fatty liver disease and viral hepatitides B and C. Alcoholic liver disease leads the way with 8 619 deaths and a mortality rate of 8.9/100 000 inhabitants in 2009, making it one of the 20 leading causes of death in the German general population. It is a growing health problem, with the number of deaths per 100 000 population doubling from 5 to 9.9 between 1980 and 2005 (49).

Only a proportion of patients affected by liver cirrhosis are eligible for transplantation or can undergo liver transplantation. In 2019, 726 liver transplants were performed in Germany after postmortem organ donation and more than 60 after living donation. Every year more than 1,000 patients will newly registered for liver transplantation in Germany (66). Liver transplant is the second most frequent transplant in the United States after kidney transplant. Since 1988, over 170,000 liver transplants have been performed in the United States. In 2019, almost 8900 liver transplants were performed in the United States. Of them, approximately 8400 were from deceased donors and 500 from live donors. Approximately 700 of these transplants involved pediatric recipients (age under 18). These numbers represent an increase with respect to the corresponding values from a decade earlier in 2009: approximately 6300 total liver transplants from 6100 deceased and 200 live donors. The number of pediatric cases was 772 in 2009 (48)

1.5 Pathology of liver grafts

Understanding the different types of injury that a liver graft is subjected to, from procurement until transplantation, is fundamental on comprehending the theoretical rationale behind the implementation of machine perfusion. The 4 phases of liver graft injury – pre-preservation, are described, and the impact that each of them – depending on its duration and severity- has on the transplantation outcome is highlighted.

1.5.1 Pre-retrieval injury

An amount of injury inevitably occurs to the liver graft before its retrieval from the donor. Brain death and sudden cardiac arrest of the donor leads to injury of the liver due to asystolic ischemia and hypoxia (67). Moreover, in DBD cases, cardiovascular instability after brain death declaration contributes to the graft injury as well (67).

Thus, the liver is unavoidably subjected to a certain amount of warm ischemia before retrieval. Besides donor type and cause of donor death, pre-preservation injury depends on several factors, including the metabolic state of the liver and duration of ischemia within the body of the donor (68). Ideally, ischemia time within the donor must be eliminated, however pre-retrieval injury is unavoidable to a certain extent (68). Injury accumulation in the liver starts already prior to graft procurement and contributes to the exacerbation of the effect of subsequent types of injury.

1.5.2 Retrieval injury

Subsequently, the liver undergoes the retrieval surgery, alongside with cannulation of the donor vessels and cold flush. During procurement, the exposure of the graft to a certain degree of mechanical injury is unavoidable. The amount of injury at this stage strongly depends on the skills and dexterity of the surgical procurement team as well as on the effectivity of the cold flush (68). Although procurement does not typically induce great damage to the liver, it is a challenging procedure and must be performed in safe way, minimizing the mechanical injury induced to the graft.

1.5.3 Cold preservation injury

Although the accumulation of liver injury begins before and during retrieval, it dramatically increases after procurement, during preservation with the golden standard method of static cold storage (SCS).

Once blood flow into the organ is ceased, the supply of oxygen and nutrients to the cells is interrupted and the graft is in an ischemic state. A series of intracellular events are initiated, that are harmful for the liver. Oxygen drives cellular respiration, which produces ATP, and when its flow stops, a shift to anaerobic metabolism occurs. Hypoxia leads to the accumulation of metabolic waste products, such as lactic acid, causing acidosis and cell swelling (28, 69) In addition, ATP depletion alters the permeability of sodium-potassium membrane pumps of cells and disrupts their function, resulting to a disruption in electrolyte gradients and membrane stability (67, 70, 71). Consequently, edema is induced in the cells and the influx of free calcium triggers a chain of reactions that promotes inflammation and leads to cellular death.

The standard preservation method of cold storage is based on establishing hypothermic conditions so as to minimize cellular metabolism. Based on the fact that metabolism is reduced by 1.5-2 times with every 10^{o}C drop in temperature (67), the

ultimate goal is to create a hypothermic environment by lowering the temperature to the point where the activity of catabolic liver enzymes during ischemia is reduced and ATP depletion is diminished. However, even at 1°C, anaerobic metabolism continues, leading to ATP depletion and eventually intracellular calcium accumulation, disturbance of the transmembrane electrochemical gradient, cell swelling and necrosis (72-74), as the result of the ischemic conditions that have been described above. This type of preservation injury is known as cold ischemia and it severly injures the graft. The deleterious effects on the liver cells strongly depend on the duration of ischemia. This type of injury aggravates during transplantation and reperfusion of the graft in the recipient, setting the stage for implantation injury as well as ischemia reperfusion injury (IRI), which are discussed in the following sections.

1.5.4 Implantation injury

The liver is exposed to further risk during implantation in the recipient before reperfusion is established. After cold storage, the graft undergoes back-table preparation, so that superfluous tissues are removed and vascular connections are reconstructed, if necessary. Then, the liver is placed in the recipient and anastomosis is performed.

During bench preparation and, especially, anastomosis, a second phase of warm ischemia takes place, as the graft temperature increases while there is no perfusion (68). This prolonged time of rewarming ischemia activates liver metabolism and increases ATP consumption; however, due to the lack of blood supply, no oxygen is delivered, and ATP cannot be synthesized. As a result, rewarming causes severe hepatocellular damage, which is evident after reperfusion and can lead to primary nonfunction of the graft (75, 76). The extent of this damage depends on the duration of both rewarming and cold ischemia that the liver is subjected to. Rewarming ischemia can elicit undesirable and catastrophic effects after reperfusion, as it exerts a serious hit on the liver particularly when cold preservation is preceded. Overall, the period of ischemia primes the tissue for subsequent damage upon reperfusion and increases the severity of ischemia-reperfusion injury (77).

1.5.5 Ischemia-reperfusion injury (IRI)

As mentioned, the injury that the liver is subjected to prior and during implantation is accumulated and aggravates once reperfusion is established in the recipient. Then, ischemia-reperfusion injury occurs, which greatly deteriorates graft quality and impacts its

viability in the recipient. Ischemia reperfusion injury is one of the major concerns in liver transplantation, as it can be detrimental for the liver and can compromise the transplantation outcome.

During ischemia, xanthine hydrogenase – a compound which normally reacts with the ATP breakdown products to produce uric acid – is converted to xanthine oxidase (78). Upon re-establishment of blood flow, oxygen is supplied again to the cells and reacts with xanthine oxidase to produce free radicals, which amplify cell damage and result in cell death (79, 80). This complex cascade of events that generates reactive oxygen species and other toxic substances upon reperfusion is called ischemia-reperfusion injury (IRI) (67, 74, 81).

IRI affects oxygen-dependent cells, including hepatocytes and cholangiocytes, which stronly rely on blood supply (82). In these cells, oxygen is necessary for mitochondrial cellular respiration to produce ATP and meet their energy demands. The extent of IRI depends on the length of ischemia – cold and warm – that the liver is subjected to as well as the background liver condition (82). Cellular damage, which occurs during both the ischemic and reperfusion phases as described, leads to cellular death via apoptosis and necrosis, which is catastrophic for the graft.

1.6 Machine Perfusion Techniques

The concept of machine perfusion instead of static cold storage has been investigated as a way to prevent ATP depletion and restore the energy reserves in the cells (74). The operational principle is based on controlled circulation of a perfusate into the organ, which provides the cells with oxygen, nutrients and metabolites, allowing the liver to maintain its metabolic function, while toxic waste products are washed away by dialysis (6, 83). Thus, the energy deficit -which is created during procurement and static cold storage- is restored, while the intrinsic repair and regenerative ability of the liver are maintained alongside with its function. The circulation of the perfusate benefits the liver's microcirculation as well, maintaining it uninterrupted; in contrast, during static cold storage, the sinusoids constrict due to hypothermia and blood flow after reperfusion is obstructed (24).

Furthermore, it should be noted that machine perfusion allows monitoring of graft function over time with detection of certain biochemical viability markers. Finally, with MP the liver is accessible for pharmacological intervention by injecting medication into the perfusate. Pharmaceutical treatment can the graft alone and be induced in any dosage,

as there is no systemic effect to other organs in the bady. In this context, machine perfusion could be beneficial in treating the donor and/or recipient by pharmaceutical administration directly into the liver.

It is, therefore, evident that MP can protect the liver from injuries and achieve improved graft quality, extended preservation periods, real-time monitoring and assessment of the graft as well as enable targeted pharmacological treatment (6, 84). As already mentioned, temperature plays a prominent role in the preservation outcome and is the distinct characteristic between the three main MP techniques: hypothermic, normothermic and subnormothermic.

1.6.1 Hypothermic Machine Perfusion

Hypothermic machine perfusion (HMP), which is typically performed between 0-12^{o}C, aims to establish an improved preservation environment compared to SCS. In theory, the hypothermic temperature slows down cellular metabolism and the circulation of the perfusate protects the liver against ischemia by delivering nutrients, eliminating toxic waste products and free radicals and -in some cases- supplying the cells with oxygen as well (84, 85). Aerobic metabolism is decreased but not ceased, and the supply of metabolic substrates and oxygen reconditions the liver cells before re-establishment of blood flow (86). ATP levels are restored, mitochondrial function is ameliorated and reactive oxygen species (ROS) release is diminished. As a result, it offers better protection against the oxidative burst and the cascade of inflammatory and biochemical reactions that create the re-perfusion part of IRI (87, 88). On the downside, the sinusoidal network of the liver is more sensitive to shear stress damage when actively perfused at cold temperatures, and therefore more injury-prone during HMP (89-91).

There have been several experimental HMP protocols that utilize different settings in terms of perfusate, oxygenation, perfusion circuit, mode of flow and even duration of perfusion. The decreased metabolic demands of the liver under low temperatures favour this versatility and offer simplicity in the design and operation of HMP devices (6).

1.6.2 Normothermic Machine Perfusion

The rationale behind normothermic machine perfusion (NMP) is the establishment of a near-physiological environment during preservation in order to maintain normal metabolic activity, viability and functionality of the liver (8, 80) .

Normal cellular metabolism is achieved by maintaining the temperature at physiological values, while supplying the necessary nutrients, metabolites and oxygen to the organ via the perfusate (92). Consequently, the energy balance is maintained in cells and ATP depletion is prevented, offering protection from ischemic injury (86, 93). In addition, maintaining the metabolic activity allows graft viability assessment during NMP, by detection and measurement of biochemical parameters, physiological functions –such as bile flow, urea production, oxygen uptake- as well as hemodynamic parameters (26, 94). Furthermore, a physiological temperature could also protect the endothelial lining from shear stress damage due to perfusate flow, which occurs at hypothermic temperatures (92).

Nevertheless, NMP is a complex technique in terms of logistics and technology and the elevated temperature induces the risk for bacterial contamination (24). Replication of a physiologic environment requires oxygenation and continuous supply of nutritional supplements in order to support the functional graft. As oxygen consumption and metabolic activity are significantly higher at physiological temperatures, any interruption or even a short lack of oxygen supply during perfusion greatly impacts the liver. Therefore, it is evident that NMP induces greater risk to the graft than HMP, as any error during perfusion can seriously damage it.

An oxygen carrier is utilized in the perfusate, such as blood, and the process becomes more technically challenging (6, 80, 93). Of note, depending on previous ischemia that the liver is subjected to, there is the danger that after perfusion with the oxygenated perfusate during NMP, ROS are released and IRI occurs (9, 15). Overall, the theoretical principle behind NMP suggests that closely mimicking physiological conditions diminishes tissue ischemia and increases metabolism, and ultimately leads to extension of the duration of preservation without jeopardizing the viability of the liver (95).

1.6.3 Subnormothermic Machine Perfusion

As a consequence of the above, the introduction of subnormothermic machine perfusion (SNMP) emerged as a promising preservation technique, which combines the advantages of NMP and HMP. It is applied over a temperature range which lies between normothermic and hypothermic perfusion. The use of a subnormothermic temperature aims to benefit the graft by a lower metabolic demand, while still maintaining a certain degree of metabolic activity (96, 97). SNMP serves as a compromise between NMP and

HMP, as the reduced metabolic activity allows for more logistic and technical simplicity to the system compared to NMP, and still offers the possibility to evaluate graft viability, even though assessment becomes more challenging (15, 98).

Sub-physiologic temperature allows for basic liver functions to be performed, and protects against ischemic injury, meaning that it can potentially increase the preservation times of the liver. Of note, thre is yet no consensus as to the ideal subnormothermic temperature that favours effective liver preservation. SNMP is the least investigated of the machine perfusion techniques, however is relied on a strong theoretical basis and holds promising applications (76, 99).

In addition to MP techniques, it is worth mentioning that there are two major approaches for ex-situ perfusion with regards to the timing in which it is applied: continuous and end-ischemic. Continuous machine perfusion is applied immediately after procurement of the graft. The liver is placed on a transportable machine perfusion device and undergoes dynamic preservation until transplantation is completed (6, 100). Application of continuous MP is possible but logistically complex in cases where procurement and implantation take place at different centres. This approach aims to eliminate the use of SCS during transportation, minimizing the ischemic time that the graft is subjected to. A novel variation of this approach involves in-situ perfusion of the liver in the donor, even before procurement, aiming to eliminate ischemia; this approach is called normotermic regional perfusion (NRP)(101) and is beyond the scope of the present review.

End-ischemic MP is performed after the organ has been initially preserved with SCS. Typically, the graft is preserved statically in an “icebox” during transportation from the donor to the recipient center (87, 102). Therefore, it is mainly preserved with SCS and is perfused for a shorter period of time before implantation. The implementation of end-ischemic perfusion aims at allowing organ assessment before transplantation, while achieving graft rewarming in a more controlled manner. Therefore, metabolic activity can smoothly increase before actual re-perfusion in the recipient, potentially minimizing implantation and rewarming injury of the graft. It is clear that this approach bears significant advantages logistically, as it is simpler and cheaper due to the absence of a MP device transport, however cold ischemia of the graft is inevitable (103).

Finally, one of the greatest advantages and arguments for machine perfusion is the ability to assess the viability and functionality of the liver graft prior to transplantation. However, there is no consensus in the literature as to which set of markers are the most

reliable for the functional evaluation of the graft during MP (94). Generally, the release of specific liver enzymes, such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT) in the perfusate, bile production as well as bile composition are utilized as markers for hepatocellular injury (94). These markers can be used as viability markers indicating hepatic or biliary function, but were shown to be inadequate in predicting the post-transplant outcome, by Watson et al. (104). Several groups have suggested that bile production is evaluated as well, alongside with its composition (92, 94); thus, pH and bilirubin levels in bile are also detected as functional biomarkers of the liver. Besides, numerous other parameters can be taken into consideration when assessing the viability of the graft, including oxygen consumption, factor V production, lactate clearance, hyaluronic acid (marker of endothelial injury), which are measured during MP . Apart from biochemical analysis of the perfusate, histological examination and hemodynamic parameters, such as arterial resistance, can also correlate to liver viability and functionality (7, 19, 105, 106). It is clear that well-defined protocols are needed, not only for the type of assessment markers, but also for determining reliable thresholds for each marker in order to safely predict the preservation outcome and predict future graft function(103).

The major endpoints used by the studies selected in the present literature review are presented in the Results section. They serve as a basis upon which the preservation outcomes can be compared for the different techniques, alongside with the different parameters, technical aspects and modalities of each MP configuration.

1.7 Machine Perfusion Technology

The ideal machine perfusion strategy would extend preservation time, resuscitate marginal grafts and facilitate reliable evaluation of liver viability and functionality. The purpose of MP devices is to accomplish successful delivery of nutrients, oxygen, metabolic and therapeutic agents to the liver graft as well as allowing reliable prediction of graft function and viability. For this reason, perfusion systems require reliable pumps, biocompatible elements on the perfusion circuit, as well as oxygenation and temperature control of the perfusion solution (107).

Thus, MP devices consist of several components, each performing a particular function. The synergy of these components, when assembled together, leads to the

dynamic preservation of the liver graft. A MP system typically consists of pumps, containers/reservoirs, collectors, a monitor or computer, a heat exchanger, an oxygenator, bubble traps and connection lines (108). The perfusate is circulated through the connection tubes due to the action of pumps, perfusing the liver, which lies in the container. The synergistic action of the rest of the elements establishes the desired conditions in terms of temperature, flow, pressure and oxygenation control, while useful markers from the liver and perfusate are analyzed and displayed externally, in a monitor. A schematic diagram of a simplified machine perfusion configuration is depicted in Figure 3.

The choice of the configuration of the device depends on the MP technique, preferred perfusion parameters, condition of the graft and specific goal of the preservation. Therefore, it is generally observed that the selection of technical units, operational parameters and setups varies between different configurations. Optimization of the preservation outcome is inextricably linked with efficient implementation of the functional elements of the device, including the pump, oxygenator, reservoir, heat exchanger, sensors and stimulators (107, 109). Currently, there is increasing research interest in the effect of each component's performance on the effectiveness of machine perfusion (107). Each of these elements offers a unique functional characteristic to the system and the selection of the type of each component is driven by the wish to achieve the best preservation outcome as well as the necessity to establish a simple, safe, versatile and cost-effective device.

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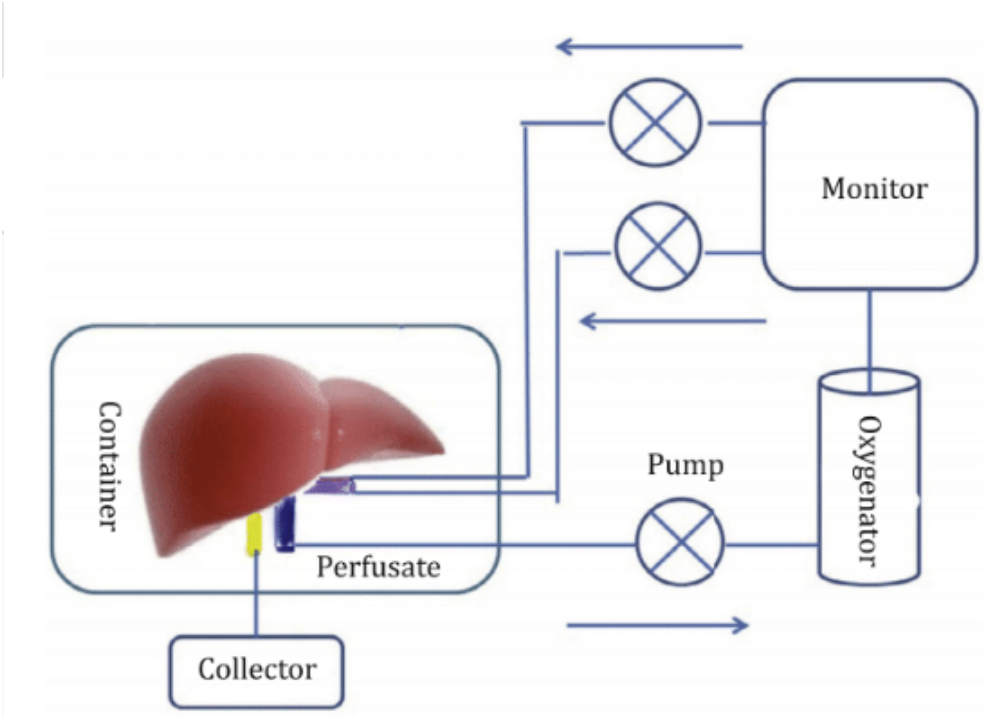

Figure 3. Schematic diagram of a simplified machine perfusion configuration. The perfusate is circulated by pumps and oxygenated by the oxygenator. Dual perfusion through both the PV and HA is performed, while bile is collected into the collector (108).

1.7.1 Pumps

There are two types of pumps utilized in MP systems: roller and centrifugal pumps. Roller pumps are positive displacement pumps that rely on peristalsis as their pumping principle, which is responsible for drawing fluid in the pump and then propelling it away. A roller generates alternate compression and relaxation in a flexible tube fitted within the rigid casing of the pump. The rotating motion of the roller compresses a section of the tube and the applied pressure pushes the fluid forward and drives it towards the discharged line, while preventing backflow (110). The priming mechanism is initiated once fluid is propelled out of the pump. The elastic tube recovers creating a negative pressure difference, which draws fluid into the suction part of the pump and the mechanism of action is repeated. The operational principle of the roller pump creates the perfusate flow in the device and its output depends on the frequency of rotation of the roller head (111).

Roller pumps are used in extracorporeal organ circuits as well as in perfusion systems due to their versatility and simplicity. Roller pumps can easily be implemented in different systemsa and they constitute a cost effective solution for the creation of flow

(110). In addition, since the liquid is completely enclosed within the tube, the danger of contamination is eliminated and the pump is considered suitable for clinical applications. The major disadvantage of roller pumps is the fact that they cause hemolysis or blood cell damage, when blood is used as perfusate (110, 112). During compression of the tube by the roller, high shear stresses are developed in the blood cells. Such high stresses on the cell membranes are also generated during recovery of the tubing after being compressed and can cause premature aging of the cells, which leads to cell clumping and even hemolysis, releasing haemoglobin into the plasma (110). As a result, the oxygen carrying capacity of the perfusate decreases over time and extension of the organ preservation time is limited. The degree of trauma is proportional to the flow rate, hence usually a low flow rate is used when roller pumps are utilized (111).

Over the last years, the centrifugal pump has emerged as an alternative, due to its lower levels of hemolysis and superior pressure-flow characteristics. The centrifugal pump is a rotary pump, the operation of which is based on the rotating motion of an impeller due to a motor (113). Fluid enters the pump from the inlet port and reaches the impeller along the rotating axis. The rotation of the impeller accelerates the fluid, which is eventually propelled outwards from the outlet, through a diffuser or volute chamber; the role of the diffuser is to reduce the velocity of outflow, by increasing the flow area along the direction of flow (113). Thus, the kinetic energy is converted into potential energy, which provides the driving pressure for the perfusate to reach the whole perfusion circuit. Centrifugal pumps are reliable, effective and simple devices that are widely used for high flow-low pressure applications. They induce minimum hemolysis and damage to cells and allow for high-accuracy control of the flow rate (114). However, they are not as versatile as roller pumps, since they are designed to function within a limited design point. The design point refers to a particular combination of flow rate and pressure; function of the pump away from these defined parameters creates adverse effects such as increased shear stress, formation of vapor bubbles and loss of pressure (110).

The choice of pumps in perfusion systems is affected by parameters such as flow rate, pressure, rate of hemolysis and operation time. The centrifugal pump is characterized by a wide range of flow rate that is dependent on pressure, whereas the roller pump displays a much limited range of flow rate, that remains unaffected by pressure (112). The degree of hemolysis induced by roller pumps is considerably higher than the centrifugal pump, and for this reason the use of roller pump is limited in applications with low flow rates and noncellular perfusion solutions, such as HMP (107). On the contrary,

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when blood is utilized as perfusate, the damage to blood cells induced by the roller pump can largely deteriorate the preservation outcome and time. Hence, in these cases, the centrifugal pump appears to be more suitable. A schematic representation of a roller and centrifugal pump are depicted in Figure 4.

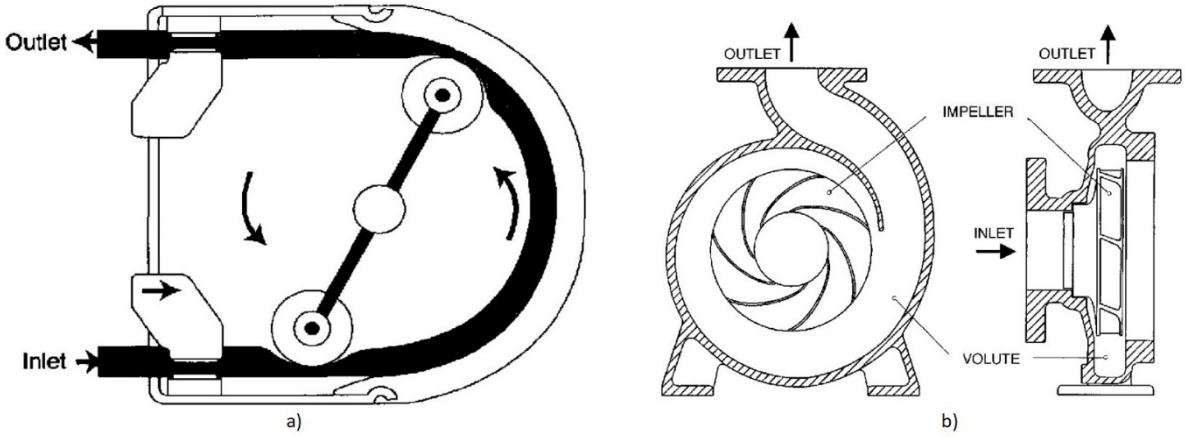

Figure 4. Illustration of a roller (a) (115) and a centrifugal pump (b) (116).

1.7.2 Perfusion Circuits

Historically, machine perfusion configurations for the liver have been experimentally designed to enable either single or dual perfusion, meaning that depending on the setup, the perfusion routes varied. Dual perfusion systems perfuse the liver through both the HA and PV, whereas single perfusion systems provide inflow through the PV only. However, as evidenced by the liver anatomy, dual perfusion matches the physiological liver flow. Thus, nowadays, dual inflow has been stablished as the standard method for liver machine perfusion, as its effectiveness over single perfusion has been confirmed.

In single perfusion settings, the perfusate enters the liver only through PV, whereas in dual perfusion settings, two branches are utilized to perfuse the organ through both the HA and PV in order to mimic the physiological dual circulation of the liver. The vessels of the liver are connected to the perfusion lines by cannulas; cannulation of the vessels is a dexterous process and must be done effectively and safely, with minimal trauma induced to the vessels (76). The effluent perfusate is collected by the IVC. In some cases, the perfusate is freely drained from the IVC into an open reservoir, while most systems employ a fully cannulated system, where the perfusate returns from the IVC to the starting point to be recirculated (80, 117).

The HA branch is designed as a ‘high pressure-low flow’ line and the PV branch as a ‘low pressure-high flow’ line in order to simulate the anatomical conditions and match the physiological characteristics of the vessels (76). Notably, the liver is a pressure-controlled organ, which means that the blood flow is determined by the pressure at the afferent vessels. The liver itself can, to a certain extent, adjust resistance to flow so that these pressures are maintained within a physiological range and prevent injury of the sinusoidal lining cells. Therefore, most MP systems are pressure-controlled (9, 118-120), meaning that a predetermined perfusion pressure dictates the flow of perfusion, which can be measured over the course of perfusion and shows the resistance of the liver. Vice versa, there also several systems in literature who operate under a flow-controlled manner (88, 121). Nevertheless, it should be noted that flow-controlled systems does not match the physiology of the liver, where inflow is pressure-guided, and can injure the liver.

Usually, the arterial line carries the perfusate directly to the HA from the pump at a high pressure. On the other hand, PV lines can perfuse the organ either directly or passively. In passive PV perfusion, the perfusate is first drained into a reservoir after propelled by the pump (118, 122, 123). This reservoir is elevated compared to the liver; from there, the perfusate passively enters the PV reaching high flow while maintaining a low pressure. Since the fluid in the reservoir is static, the perfusion pressure only depends on the height level of the reservoir, the density of the perfusate and the acceleration of gravity:

$$P = \frac{F}{A} = \frac{F \cdot h}{A \cdot h} = \frac{m \cdot g \cdot h}{V} = \rho \cdot g \cdot h$$

, where ρ is the density of the perfusate, g the gravitational constant and h the height of the reservoir. Passive PV inflow protects the liver parenchyma from injury and allows the graft to be perfused exactly to its natural maximum volume. The PV flow is here the result of a constant physiological applied PV pressure. Alternatively, the perfusate can be carried to the PV directly by a pump, the settings of which are adjusted to provide near-physiologic pressure or flow. The HA and PV pressures are generally selected within physiological values, around 60 mmHg and 2-6 mmHg respectively (122).

One of the major challenges in MP systems is the provision of physiological hemodynamics while maintaining simplicity (120). The continuous flow created by the passive drainage also emulates the physiological flow of blood into the PV, whereas active PV perfusion can either be performed with pulsatile or non-pulsatile flow.

Likewise, the flow in the arterial line can either be pulsatile, simulating physiological conditions, or continuous. It is based on the pump settings and is one of the many design decisions. Nonetheless, the implementation of two separate perfusion lines enables the selection of different flow-pressure characteristics for each route in the same dual perfusion MP circuit (117). The desired pressure (or flow) can be achieved in various ways. Commonly, adjustable C-clamps are employed along the lines to redistribute resistance (92); thus, targeted pressures can be achieved by adjusting the pump's RPMs and C-clamps tightness. In addition, MP devices in which 2 parallel systems can operate independently to provide autonomous venous and arterial regulation have been developed by the Groningen and Barcelona groups (118, 119). Finally, Borie et al. employed a different configuration, where the portal vein was fed directly from a centrifugal pump. An additional roller pump was added in series and distal to the main centrifugal pump of the system to increase the HA pressure (124).

Since there is no consensus as to which model achieves the optimal preservation outcome, there is a relatively large freedom in the design choices regarding MP systems. There is a tradeoff between complexity and performance and for the device to be simple and cost effective, it is necessary that each addition substantially contributes towards an improved preservation outcome.

1.7.3 Oxygenation

In an attempt to ameliorate ischemic and reperfusion injury, liver MP models deliver oxygen to the liver cells during preservation, hence they utilize an oxygenated perfusate. The degree and method of oxygenation is dependent on the temperature, perfusate and goal of the preservation. For instance, in cases where the preservation temperature is high and the graft is metabolically demanding, a higher degree of oxygenation is required to prevent anaerobic metabolism and graft injury (80). With regards to HMP, the first trials included non-oxygenated perfusates based on the assumption that hypothermia slows the metabolism and minimizes oxygen requirements. Since the first report of hypothermic oxygenated perfusion (HOPE) of the liver (125), which showed promising results in favor of oxygenation, there have been several HMP models in which an oxygenated perfusion medium is implemented aiming to restore the intrahepatic energy levels (126-128). Indeed, there has been evidence that justify the utilization of oxygenated perfusates and indicate that oxygen has beneficial effects on the preservation and transplantation results (125, 129, 130).

Oxygenation of the perfusate can be achieved passively or actively. Passive oxygenation is accomplished either by dissolving oxygen in the perfusate (131) or by exposing the tube to ambient air within the perfusion chamber and relying on the gas interchange between the perfusate and the air (121, 132). It has been shown that ambient oxygen diffusion leads to relatively elevated and stable oxygen levels in the effluent perfusate during perfusion. Passive oxygenation is generally sufficient for hypothermic temperatures, however when higher levels are needed, active oxygenation is needed.

Active oxygenation of the MP perfusate is achieved by implementing an oxygenator into the system. The type of oxygenator that is typically used in MP devices is the membrane oxygenator and is placed after the pump in the MP configuration. In this case, the perfusion line passes through the oxygenator, which is usually continuously flushed with a gas mixture of 95%O_2/5%CO_2(132). The perfusate and gas stream are separated by a semi-permeable membrane, which allows gas oxygen exchange between the perfusate and the feed stream, and prevents contact between the two stream lines (133). Thus, according to Henry's Law, $P = kC$, where P and C is the gas pressure and concentration respectively, and k is Henry's Law Constant of the gas, higher oxygen concentration in the gas mixture leads to a greater amount of dissolved oxygen in the liquid perfusate (133, 134). Furthermore, in cases where blood is used as a perfusate, CO_2 is also transferred from the blood to the gas mixture oxygenator, based on the same gas exchange principle (133).

The gas interchange that occurs in the oxygenator is based on diffusion, which dictates that gas molecules are transferred from an area of higher concentration to an area of lower concentration. The difference in concentration is the driving force of diffusion, and gas exchange continues until concentration equilibrium is reached. The diffusibility of each gas depends on the gas itself, the material to be crossed as well as the temperature. However, in membrane blood oxygenators it was observed that the transference of gases was more complicated as there were several obstacles that needed to be overcome. These obstacles include transfer of O_2 through the membrane, dissolution in the plasma, and entering into the cytoplasm of red blood cells to bind with hemoglobin, the major oxygen carrier in the blood. On the other hand, the diffusion of CO_2 from blood to the feed stream is much simpler, as CO_2 is around 20 times more soluble than O_2, and its transfer only depends on the partial pressure difference (133). Apart from the difference in partial pressure, the diffusion of O_2 in the oxygenator strongly depends on the membrane's material, thickness and porosity.

In order to improve the efficiency of gas interchange, hollow fiber oxygenators were investigated. Nowadays, most commercially available oxygenators have been developed for blood oxygenation and they are characterized by the hollow fiber technology. In this approach, the gas stream of the oxygenator flows through hollow fiber capillaries, and comes in contact with a microporous membrane that separates it from the blood, which flows on the external side. Thus, the gases do not dissolve in the material of the membrane, but pass through the micropores (133). This method has increased the surface contact between blood and the membrane, hence decreasing the overall surface area and size of the oxygenator and led to more efficient gas exchange. In addition, it has been proven to be more suitable for blood preservation and protection against hemolysis (134). Despite the fact that hollow fiber oxygenators are mainly used for blood oxygenation, their operation has also been tested with acellular perfusion solutions, yielding satisfactory results(118) and are the most common type of oxygenator utilized in liver MP devices.

The main problem in perfusate oxygenation is the need to ensure that oxygen is successfully carried and administered into the liver cells. When blood is the perfusion medium, oxygen is carried by hemoglobin and transported to liver cells, achieving effective perfusion. In the case of acellular perfusion solutions, oxygen can either be dissolved or an artificial carrier can be used (135, 136). The need for an oxygen carrier becomes pore-prominent when higher operating temperatures are used, thus an increased amount of oxygen needs to be administered in the perfusate (133, 137). Ideally, oxygen carriers would lead to replacement of blood-based perfusion by acellular perfusates and potentially protect against disadvantages such as immune-mediated phenomena, hemolysis and transmission of infections as well as logistical difficulties. However, as of now, there has been no report of successful implementation of artificial oxygen carriers in the perfusate.

1.7.4 Bubble Traps

One of the issues that need to be addressed in machine perfusion systems is the creation and accumulation of air bubbles due to the constant flow and recirculation of the perfusate. The buildup of bubbles in the perfusate can impede the flow and damage the cells by inducing shear stress at the liquid-gas interface (138). In addition, the surface tension that is created hinders the task of removing the generated air bubbles, while an increase in the flow rate to remove the bubbles would cause further damage to the cells. Therefore, bubble traps are implemented in several machine perfusion systems(98, 139)

to effectively eliminate air bubbles from the perfusate and ensure smooth circulation without injuring the liver cells.

Typically, a bubble trap is comprised of an inlet, a microporous membrane and an outlet. The trap is connected with the perfusion line of the system and the perfusate enters the trap through the inlet, and then returns to the perfusion line through the outlet of the trap. Within the bubble trap case, the perfusate comes in contact with the permeable membrane and the bubbles are forced to move from the solution to the back outlet of the trap due to the pressure difference (140). Consequently, when the perfusate exits the bubble trap, the air bubbles are eliminated. In addition to this passive mode of operation, the vacuum outlet of a pressure pump can be connected with the bubble trap in order to maximize the generated pressure difference and, consequently, the efficiency of the trap (140). However, the trap typically displays satisfying and effective performance with the passive mode of operation. Therefore, this mode is generally preferred in machine perfusion systems due to its logistic simplicity combined with adequate efficacy.

1.7.5 Sensors

Real-time measurements during machine perfusion provide continuous feedback on the system's operation and enable monitoring of the liver's viability and reaction to the perfusion. Hence, several types of sensors that measure perfusion parameters -such as pressure, flow, temperature and pH- can be implemented in machine perfusion devices. In certain cases, optical oxygen and glucose sensors are installed as well indicate the status and functionality of the liver during perfusion (107, 141). In general, the most common types of sensors that are implemented in machine perfusion systems are pressure and flow sensors.

Pressure sensors are typically placed in the inflow line of the liver, immediately prior to the cannulas of the portal vein and hepatic artery. Maintaining a constant and near-physiological perfusion pressure in the portal vein and hepatic artery is of great importance, as it allows for efficient perfusion without inducing damage to the vascular endothelium of the liver (19). Generally, different types of pressure sensors are implemented in different machine perfusion devices. Accurate measurements, that keep the configuration safe and as simple as possible, are the required characteristics of the utilized sensors. Pressure transducers, which convert the measured pressure into electrical signal and transmit it to a monitor, allow real-time pressure monitoring in many machine perfusion systems (92, 95, 121, 141, 142). A catheter placed in the perfusion line is connected with the

pressure transducer, which transfers the pressure information to the monitoring system. The operation of the transducer is typically based on the principle of capacitive measurement. The capacitance transducer is comprised of two adjacent metallic plates; the static plate, which is fixed within the transducer casing and the elastic metallic diaphragm, which can move. The plates are separated by a dielectric medium. Capacitance is defined by:

$$C = \varepsilon_r \varepsilon_0 \frac{A}{d},$$

where ε_r is the dielectric constant of the medium, ε_0=8.854·10^{-12} F/m is the electric constant, A is the area of the plates and d is the distance between the plates. Thus, when a pressure difference causes the diaphragm to deflect towards the static plate, the capacitance changes as a function of the pressure difference (143). As the capacitance can be calibrated into voltage, proportionally to the applied pressure, the pressure difference can be measured. An illustration of a capacitance transducer and its aforementioned elements is depicted in Figure 5.

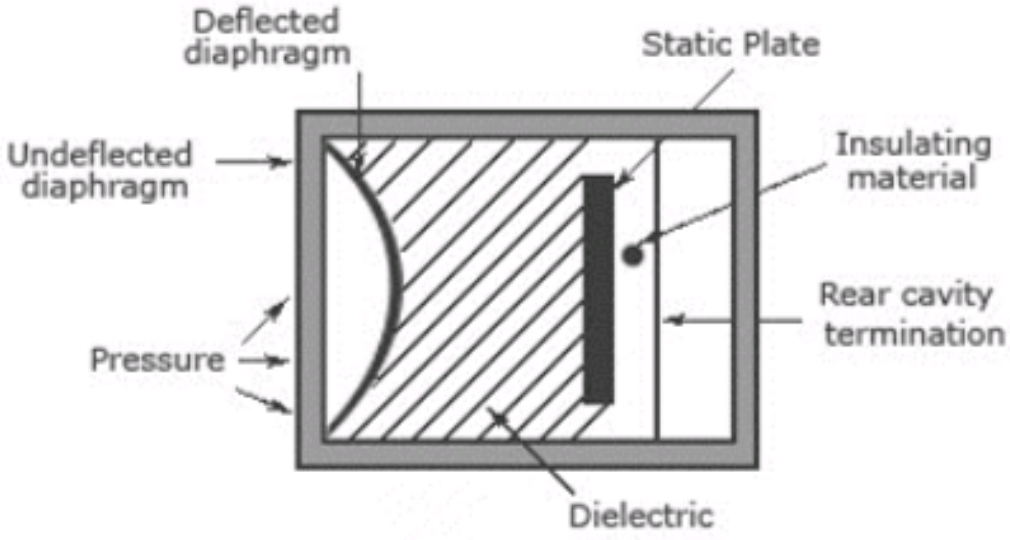

Figure 5. Schematic representation of a capacitance transducer and its functional elements (144).

Such sensors can provide reliable measurements with good sensitivity in the desired pressure range for machine perfusion applications. In addition, they display versatility, chemical stability and mechanical simplicity and can be used for both absolute and differential pressure measurements (145). In several configurations, the pressure at the portal vein is measured by connecting a water column to the portal vein catheter (146,

147). The utilized pressure measurement equipment can either be autoclavable or single-use to ensure sterility. In automated pressure-controlled perfusion systems, the transducer can be connected to the main pump of the device in order to provide on-line feedback for the pressure regulation (141, 148).

Alongside with pressure, perfusate flow is one of the most significant and commonly measured parameter for machine perfusion. Flow and pressure are used to estimate the vascular resistance of the liver, which gives information on the micro-vascular integrity of the organ and is considered as one of the indicators for the quality and effectiveness of the perfusion (90, 149, 150). Flow measurement in machine perfusion setups is accomplished by means of flow probes (71, 92, 126, 151), which are mainly ultrasonic-based. These flow meters utilize sound waves in order to calculate the velocity of the perfusate. When the sound waves are reflected in the flowing perfusate, the frequency increases alongside with the velocity of the perfusate, according to the Doppler effect, and the signal from the reflection of the wave is processed in order to calculate the flow rate (152). Ultrasound flow meters offer simplicity, easy use and are widely used as clamp-on tubing sensors. However, any changes in temperature, pressure or perfusate can affect the accuracy of the measurement (150).

1.7.6 Encapsulation and Temperature Control

Once the perfusion circuit is assembled, it is placed in a chamber, which isolates and protects the graft from the external environment. The purpose of encapsulation is to offer sterile environment, which includes defined and/or physiological levels of humidity and temperature, that will allow perfusion of the liver to be successful (107). The perfusion chamber should provide adequate space for the user to be able to intervene if necessary. Finally, sterility is significant, as the graft should be protected from any bacterial infections.

There have been several experimental machine perfusion setups, which encapsulate the perfusion circuit within a Plexiglas or acrylic cube (153). Such cubes offer adequate thermal insulation from the external environment, and its transparency allows the experimenters to continuously observe and monitor the perfusion. In addition, such materials are easily processed and access points can be created, through which the operators can intervene in the circuit.

Since temperature is one of the most crucial aspects of machine perfusion, devices usually utilize automated temperature control systems to ensure that temperature is kept

at the targeted level (71, 92, 98, 126, 151). In normothermic and subnormothermic devices, the simplest way for temperature regulation is to place a temperature probe within the chamber and connect it to a heating device. The desired temperature is set and selected on the controller of the probe-heating device configuration, and the temperature of the devices is continuously and automatically regulated (71, 92). Alternatively, the perfusate can be maintained in a constant temperature by using heat exchangers, which can be connected to a thermostat or thermoelectric pump. Again, a temperature sensor connected to the pump enables real-time control of the temperature (151, 154). In addition, a water-jacketed apparatus can be employed to regulate the temperature (98). The device is surrounded by a water-filled sheath, which allows water to flow and circulate. As water is pumped to an external heating or cooling device, accurate temperature control of the device is achieved (155). Such temperature-regulating systems can be used in versatile experimental setups, where perfusion in a wide range of temperatures needs to be tested

Therefore, it is evident that protective casing and precise temperature regulation mechanism are needed to complete a machine perfusion device. For clinical application, the whole device should ideally be transportable and energetically autonomous (135).

1.7.7 Perfusion Solutions

With regard to the types of perfusates, the most commonly used preservation solution for HMP of the liver is the University of Wisconsin solution (UW) or UW-gluconate (UW-G) or UW-MP solution (24). Its main components are: gluconate, a metabolically inert substrate which gives the solution the desired osmotic concentration; glutathione, an important supplement in order to reduce the generation of ROS; potassium, sodium and glucose; adenosine, ATP precursor in order to offer sufficient energy levels to the liver; finally, hydroxy-ethyl starch (HES) is added as a colloid to increase the oncotic pressure of the solution (24, 84).

In addition, Vasosol, an HES-based solution similar to UW has also been developed and used in several setups (28, 121). Despite their extensive use in experimental liver MP trials, HES-based preservation solutions display certain disadvantages in MP. Namely, the presence of HES leads to high viscosity of the perfusate, especially at low temperatures, and can cause clotting of the tissue with the solution. Therefore, there is the possibility of red blood cells (RBC) aggregation upon reperfusion, which eventually impedes the flow of blood (156, 157). Moreover, the high potassium content of such solutions is associated with depolarization of the cells and activation of intracellular voltage-

dependent channels (86).Therefore, certain modifications or additions can be applied to the perfusate in order to encounter the disadvantages and match the metabolic needs of the graft as well as the goal of the preservation.

Schlegel et al. successfully performed HMP in porcine livers using a modified starch-free UW solution to ameliorate the high viscosity (158). As an alternative, other preservation solutions, which do not contain oncotic substrates have been developed and used, such as Celsior and Histidine-tryptophan-ketoglutarate (HTK). Notably, polyethylene glycol (PEG) offers similar oncotic pressure to HES without increasing viscosity and has been used in Institute Georges Lopez (IGL-1) solution and Polysol solutions (28). The abovementioned perfusion solutions are presented and compared in Table 1.

Table 1. Overview of major perfusion solutions

HES-based

Solution	*Main Components*	*Advantages*	*Disadvantages*
UW	Gluconate, glutathione, potassium, sodium, glucose, adenosine, hydroxyethyl starch (HES)	1.Effective preservation 2.Matches the liver metabolic demands	1.High viscosity (tissue clotting) 2.RBC aggregation
Vasosol (28, 121)	Gluconate, potassium, sodium, adenine, nitric oxide donors, nitric antioxidants, vasodilators, hydroxy-ethyl starch (HES)	1.Adequate metabolic support 2.Better amelioration of IRI than UW	1.High viscosity 2. Increased aggregation of RBCs

No oncotic substrates

Solution	*Main Components*	*Advantages*	*Disadvantages*
Celsior (159, 160)	Potassium (decreased), calcium, magnesium, histidine, mannitol	1. Low viscosity, higher flushing rate	1.Risk for primary dysfunction, initial poor function or primary nonfunction

		2.Improved vascular endothelial injury	
HTK (161, 162)	Histidine, mannitol, a-ketoglutarate, tryptophan, potassium, sodium	1. Low viscosity, improved washout of blood elements 2. Improved biliary protection 3.ffective for short preservation time, more suitable for LDLT	1.Risk for primary dysfunction, initial poor function or primary nonfunction 2.Not great for long-term preservation

PEG-based

Solution	*Main Components*	*Advantages*	*Disadvantages*
Polysol (163)	Glutathione, adenine, adenosine, glucose, sodium, potassium, amino acids, vitamins, polyethylene glycol (PEG)	1.Low viscosity 2.Improved quality of steatotic livers 3.Effective as washout solution	1.Unclear effect on long-term preservation
IGL-1 (86, 164)	Glutathione, adenosine, potassium, sodium, magnesium, lactobionic acid, raffinose, polyethylene glycol (PEG)	1.Low viscosity 2.Protection of hepatic microcirculation against IRI	1. Risk for primary nonfunction

Machine perfusion devices are complex systems, consisting of a great number of smaller parts and displaying a high degree of technology. The discussed elements perform the major functions required for the application of machine perfusion and can be found in almost every configuration. Based on the goal of the perfusion, more elements can be added to the system and many different assemblies can be created. However, with each addition the degree of complexity induced to the system is increased, and a tradeoff between simplicity and functionality is eventually achieved.

Despite the fact that most developed liver machine perfusion systems in the literature are experimental and custom-made, there are commercially available devices, such as the Liver Assist (Organ Assist) (165), LifePort Liver Transporter (Organ Recovery Systems) (166), TransMedics Organ Care System (TransMedics) (167) and Organ Ox Metra (Organ Ox) (168). Each of these devices is used for a machine perfusion at a specific temperature and for certain duration of perfusion. These systems possess the functionality required for effective perfusion at the preset conditions, while also being user-friendly and transportable, so that they can be used under clinical circumstances.

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2. Objectives

Ex Vivo Liver Perfusion provides a new promising preservation approach which might replace cold storage in the future. Beside this it offers the possibility of organ assessment and repair prior to transplantation. The aim of the experimental work in this thesis is to establish a large animal model to investigate the impact of different parameters of perfusion (temperature, perfusion solution) on the liver. The first study is designed to investigate the effects of normothermic perfused preservation in a clinically relevant model of combined warm and cold ischemic injury. We determined whether hepatocyte and bile duct injury can be improved by ex vivo normothermic perfused preservation (NEVLP) in comparison to cold static storage.

The second study compares cold storage (CS) with combined CS and subnormothermic ex vivo liver perfusion (SNEVLP) for the preservation of donation after cardiac death (DCD) liver grafts in a model of pig liver transplantation. It outlines the effects of SNEVLP in DCD grafts on hepatocyte, sinusoidal endothelial cell (EC), and bile duct injury after transplantation.

The purpose of the present literature review is to investigate which set of perfusion settings has been primarily applied in each liver machine perfusion technique, and examine which combination yields the best preservation outcome, especially on long-term liver preservation. Therefore, the research question is defined as: “Which settings – in terms of perfusate, mode of flow and perfusion route – are utilized in different liver machine perfusion techniques, how do they affect the preservation outcome and which combination would be optimal for long-term preservation?”. In addition, the review aims in presenting liver machine perfusion devices from an engineering standpoint, describing and analyzing the technical aspects, major components and their function in the perfusion configuration. Overall, the current state of both clinical and technical knowledge on liver machine perfusion is given and the major findings are highlighted.

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3. Methods

3.1 Normothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

3.1.1 Animals

Male Yorkshire pigs, 30–35 kg, were utilized for this study. All animals received humane care in compliance with the ‘‘Principles of Laboratory Animal Care’’ formulated by the National Society for Medical Research and the ‘‘Guide for the Care of Laboratory Animals’’ published by the National Institutes of Health. The Animal Care Committee of the Toronto General Research Institute approved all studies.

3.1.2 Acellular normothermic ex vivo perfusion circuit

The perfusion circuit consists of a hard-shell reservoir (HILITE MVC1630, MEDOS AG, Stolberg, Germany), a centrifugal pump (Revolution, Sorin Group USA, Inc., Arvada, CO, USA), hollow-fiber oxygenator/heat exchanger (Vision Hollow Fiber Oxygenator, GISH Biomedical Inc., Rancho Santa Margarita, CA, USA) and a leukocyte filter (LeukoGuard LG Arterial Filter, Pall Corp., Port Washington, NY, USA; **Figure 6**).

Acellular NEVLP was performed with 3 L of Steen solution (Vitrolife, Denver, CO). Steen is a buffered extracellular solution containing dextran and albumin at an optimized colloid osmotic pressure. Additives to the solution include cefazolin 500 mg, hourly sodium-heparin (1000 U/h) and a continuous infusion of epoprostenol(4 mg/h). The perfusate did not contain any serum or blood components, or oxygen carriers.

The perfusate was warmed up to normothermic temperature (38°C, pig core temperature) and 100% O_2 was connected to the oxygenator at a sweep of 1L/min. Perfusion through the hepatic artery (HA) was started at a pressure of 60 mmHg, while the portal vein (PV) pressure was adjusted to 10 cmH_2O pressure. During NEVLP the arterial flow was 365 ± 85 cc/min and the portal venous flow 950 ± 110 cc/min. Ultrasonic flow probes were placed on the HA and PV circuit outflows to measure flow. The mean pH of the perfusate was 7.32 without requiring bicarbonate.

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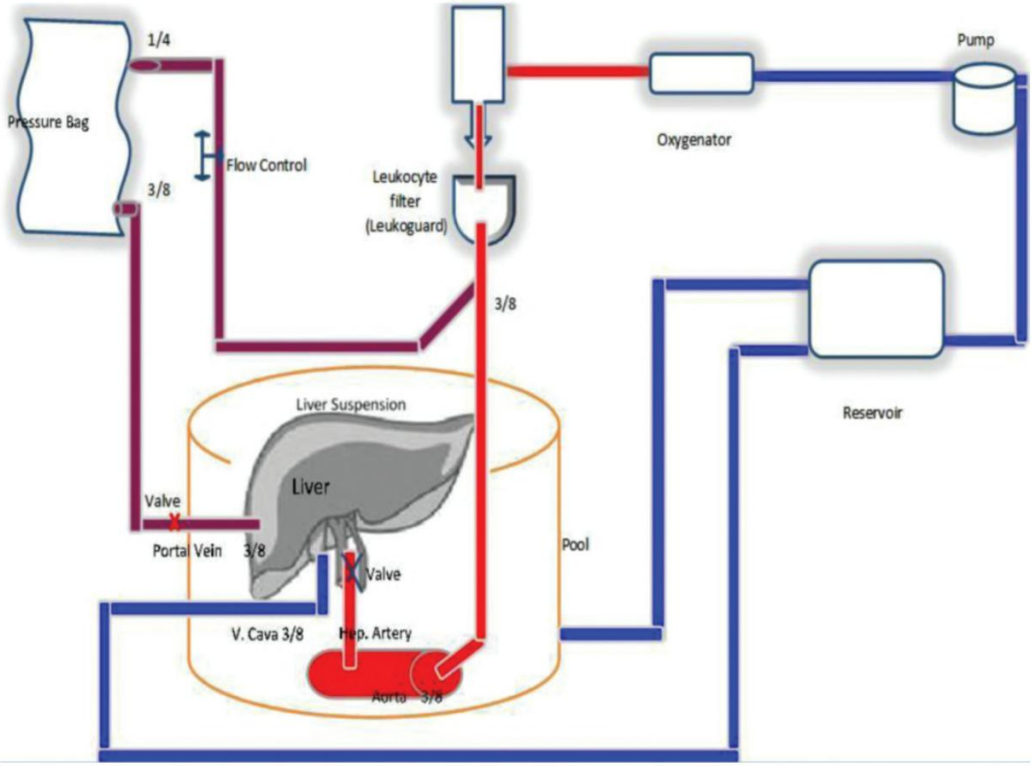

Figure 6: The circuit consists of a centrifugal pump, an oxygenator and a heater, resulting in an oxygen tension of 600 mHg at 38°C. The perfusate passes through a leukocyte filter to remove inflammatory cells. The liver is perfused via the hepatic artery with a pressure of 60–80 mmHg resulting in an arterial flow of 250–400 mL/min. The portal vein is perfused by gravity with a pressure of 4–8 mmHg resulting in a portal flow of 900 mL/min.

3.1.3 Study design

A model of DCD liver retrieval was designed by inducing 1 h cardiac arrest through exsanguination at the end of the vascular dissection during the organ retrieval procedure. Briefly, the donor pigs were sedated with Ketamine (0.2 mg/kg), Atropine (0.08 mg/kg), and Midazolam (0.06 mg/kg) and induced with Isoflurane (5%). The pigs were intubated and ventilated with 100% oxygen and 3% isoflurane for the duration of the procedure. Laparotomy was then performed and the infra-renal inferior vena cava (IVC), aorta, renal arteries, superior mesenteric artery, celiac trunk and HA, PV, suprahepatic IVC, bile duct and the sub-diaphragmatic abdominal aorta were then dissected free and encircled. The gallbladder was removed from the liver. The donor pigs received 1000 IU of heparin per kg body weight 5 min prior to cardiac arrest. Following 60 min of cardiac arrest the liver was flushed with cold UW solution and stored on the ice.

Three groups of 6 animals each were used: In the NEVLP group, the liver was stored for 4 h on ice, followed by NEVLP for additional 8 h (total preservation time 12 h). In the

control groups the grafts were continuously stored for 4 h (A) or 12 h (B) on ice. At the end of the preservation time, all three groups were perfused ex vivo with diluted pig blood with a hematocrit of 15% for an additional period of 12 h as a surrogate model of transplantation. The leukocyte filter was removed from the circuit during blood reperfusion to allow blood-derived leukocytes to initiate reperfusion injury.

In a second model orthotopic pig liver transplantation was performed using a passive portocaval shunt as previously described (169). For the transplant experiments 60 min of warm ischemia plus 4 h cold storage plus 4 h normothermic perfusion (NEVLP group) was compared with 60 min of warm ischemia and 8 h of cold storage.

Parameters of hepatocyte function and injury:

Alanine aminotransaminase (ALT) was measured hourly after blood reperfusion in the perfusate as a marker of hepatocyte injury. Liver necrosis was assessed by H&E and trichrome staining of the liver section collected at the end of blood reperfusion (12 h). BUN production was measured hourly in the perfusate as a marker of liver synthetic function. Oxygen extraction of the liver from the perfusate was measured hourly in the perfusate as a marker of liver metabolism.

Markers of bile duct injury and function:

Bile production was monitored hourly during the blood perfusion period. The bile content was analyzed for bilirubin, phospholipid, bile salts and LDH concentrations (170). Bile salts were measured by an enzymatic method (3-alpha-hydroxysteriod dehydrogenase) with colorimetric detection (Trinity Biotech, Bray, Ireland) as described by Mashige et al. (171). Total bile salts including cholate, chenodeoxycholate, deoxycholate, lithocholate and the conjugated forms were measured. Bile phospholipids were measured by an enzymatic method (Phospholipase D) with colorimetric detection (Wako Diagnostics, Richmond, VA). Total phospholipids including lecithin, lysolecithin and sphingomyelin were determined. Bile duct viability was investigated by trichrome staining on histology.

Calculation of oxygen extraction:

The reverse Fick Equation: $VO_2 = Q\ (CaO_2 \times CvO_2)$ was utilized to calculate oxygen consumption (VO_2). Pump flow rate was utilized as cardiac output 2 (Q) and oxygen content of the perfusate pre liver (CaO_2) and post liver (CvO_2) was calculated using the oxygen content equation: $CxO_2 = ([Hb] \times 1.36 \times SxO_2) + (0.0031 \times PxO_2)$. Hemoglobin concentration ([Hb]) of 8 g/dL was used for blood reperfusion. Kelman's equation (172) was

utilized to calculate hemoglobin saturation (SO_2) of the blood perfusate at the corresponding PO_2.

Computer tomography (CT) angiography:

At the end of the 12 h blood perfusion, each liver was placed in CT- compatible acrylic container filled with 0.9% saline. CT angiography was performed with a 320-slice dynamic volume CT (Aquilion One, Toshiba Medical Systems, Ottawara, Japan) using 16 cm detector coverage with 0.5 mm primary slice thickness and 0.5 mm reconstruction interval resulting in 320 axial images per volume. The scout-view in anterior–posterior and lateral direction was used to define the scan range. A single 16 cm volume was acquired prior to contrast material injection to confirm central position of the liver and to ensure almost complete coverage. Imaging was performed with tube voltage of 100 kV, 300 mA tube current and 0.5 s gantry revolving time. The HA was connected to a dual head power injector. A total of 60 mL of 85% diluted nonionic contrast material (370 mg iodine/mL, Ultravist, Bayer-Schering, Berlin, Germany), was injected at a flow rate of 2 mL/s. Continuous scanning over a time of 30 s was started simultaneously.

Reconstructed CT images were transferred to a commercially available stand-alone workstation (Toshiba Medical Systems) and data were processed by an experienced radiologist blinded to the liver's group assignment (PR). The volume with the highest vascular contrast was defined and a maximum intensity projection (MIP) with a slice thickness of 40 mm performed. The Hounsfield units (HU) were measured in two regions, with best and worst arterial branching. We performed measurements in three regions of interests (ROI) measuring 80 mm^2 centrally, in mid and peripheral position. Mean density, measured in HU, were obtained.

Histology

After 12 h blood reperfusion biopsies were stored in 10% formalin overnight and then exchanged for 70% ethanol until paraffin embedding. Sections 5 µm in thickness were then cut. Histology was evaluated by a blinded liver pathologist (A.O.). Hematoxylin and eosin (H&E) and trichrome staining, were performed (173). Hepatocyte necrosis was determined in H&E stained tissue sections by a point counting method using a semi-quantitative scale as previously described (75, 173, 174). Thirty random fields were investigated per slide to determine the area of necrosis. In this study, only Grade 3 injury with destruction of hepatic cords was counted as necrosis.

Statistical analysis

The data were analyzed with the SPSS 16 statistical package (Chicago, IL, USA). Analysis of variance (ANOVA) was used for the comparison of continuous variables, while a Fisher's exact test was applied for categorical outcome. The results are presented as mean ± SD and were considered significant at the level of $p < 0.05$.

3.2 Subnormothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

3.2.1 Animals

Male Yorkshire pigs (30-35 kg) were used for this study. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The animal care committee of the Toronto General Research Institute approved all studies.

3.2.2 Subnormothermic Ex Vivo Perfusion Circuit

The perfusion circuit consisted of a hard-shell reservoir (Cardiotomy reservoir 2811), a centrifugal pump (Rotaflow centrifugal pump), a hollow-fiber oxygenator/heat exchanger plus a hard-shell reservoir (Quadrox-I Adult HMO70000 and VHK 2001; all from Maquet, Hirrlingen, Germany), a hollow-fiber dialyzer (NR16, Fresenius, Bad Homburg, Germany), and a leukocyte filter (LeukoGuard LG arterial filter, Pall Corp., Port Washington, NY; **Fig. 7**).

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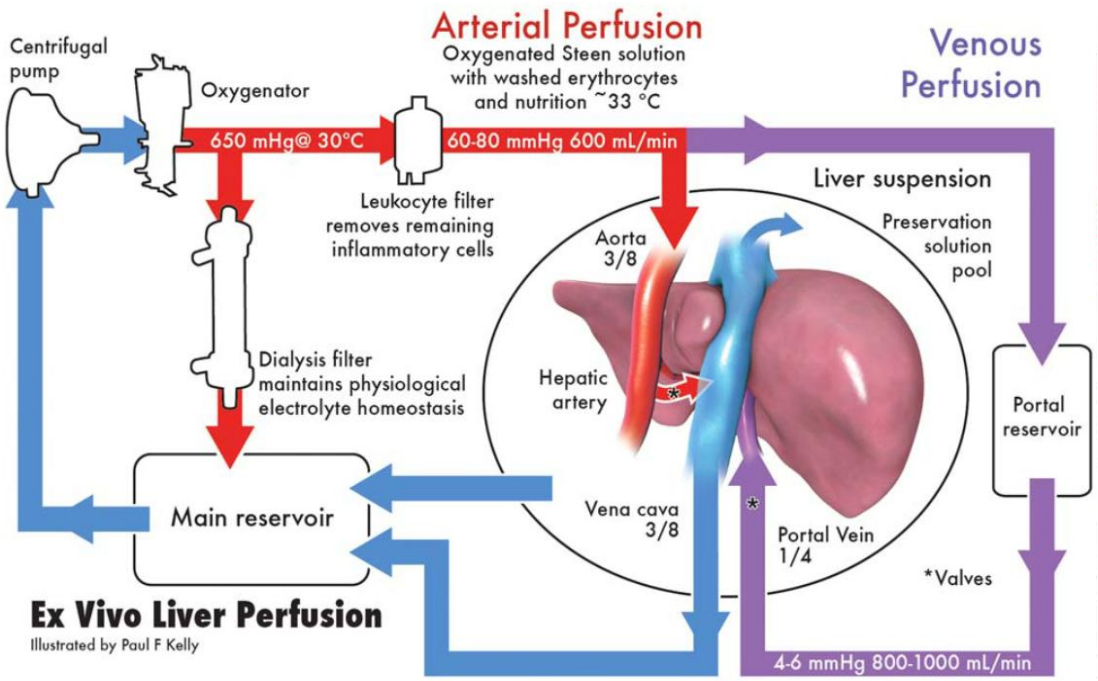

Figure 7. The circuit's perfusate is driven by a centrifugal pump at 1700 to 2000 rounds per minute. An oxygenator, including a heating-cooling unit, saturates the perfusate to an oxygen pressure of 650 mm Hg at 33°C. The perfusate passes through a leukocyte filter to remove inflammatory cells. The liver is perfused via the hepatic artery with a pressure of 60 to 70 mm Hg, and this results in an arterial flow of up to 500 mL/minute. The portal vein is perfused by gravity with a pressure of 4 to 8 mm Hg, and this results in a portal flow of 900 to 1100 mL/minute. The liver is placed on a heatable water bath detached by a sterile organ bag. The effluent returns in a closed system via the upper and lower vena cava back to the main reservoir.

SNEVLP was performed with a 3-L Steen solution (XVIVO Perfusion, INC., Goteborg, Sweden) plus washed erythrocytes to achieve a hematocrit of 10% to 12%. The erythrocytes were washed 3 times and passed through a leukocyte filter to avoid contamination with serum and to remove unwanted proinflammatory cells. The Steen solution is a buffered extracellular -type solution containing dextran and albumin to provide an optimized colloid osmotic pressure. The perfusate contained heparin (10,000 IU; Sandoz Canada, Boucherville, Canada) to prevent clot formation from residual coagulation factors. For metabolic supplies, the perfusate comprised an amino acid concentrate (50-mL bolus plus 8 mL/hour; 4.25% Travasol, Baxter, Hamilton, Canada), Ringers lactate in D5W (150 mL; Baxter), and insulin (40 IU/ hour; Humulin R, Eli Lilly, Indianapolis, IN); cefazolin (1 g; Pharmaceutical Partners of Canada, Richmond Hill, Canada) and metronidazole (500 mg; Baxter, Toronto, Canada) were added to prevent bacterial contamination. To

improve flow properties by vasodilatation, a bolus of BQ 123 (1.7 g; AG Scientific, Kelowna, Canada) and a continuous infusion of alprostadil (250 mg/3 hours; Pfizer, Kirkland, Canada) were used. Acetylcysteine (6 g; Sandoz Canada) was added for its free radical2scavenging properties. Notably, none of the perfusate components were renewed during perfusion. The perfusate did not contain any serum parts. A gas composition of 95% O_2 and 5% CO_2 was connected to the oxygenator at a sweep of 2 L/minute. Additionally, active gaseous components were added: CO (1000 ppm; Praxair, Burlington, Canada) for its vasodilatative and anti-inflammatory properties (43, 175, 176) and sevoflurane (1.5%; Abbott, Saint-Laurent, Canada) for its protective properties for ECs (177). Both were also administered through the oxygenator. The liver was perfused at 33°C with a continuous flow. Dialysate was produced with a standard concentrate (D12188; Baxter), double-reverse-osmosis water, and sodium bicarbonate (Hospira, Montreal, Canada). Five hundred milliliters of the ready-to-use dialysate was perfused through the dialyzer per hour. The objective for the dialysis unit was to maintain the electrolyte concentration of the perfusate stably. The excreted dialysate was replaced 1:1 with dialysate fluid containing the desired electrolyte concentration. Perfusion through the hepatic artery was set at a pressure of 60 to 70 mm Hg, and this resulted in a flow of up to 500 mL/minute. The portal vein pressure was adjusted between 4 and 8mm Hg, which corresponded to a flow of 900 to 1100 mL/minute. Ultrasonic flow probes (HT 110 flow meter, Transonic Systems, Ithaca, NY) were placed on the hepatic artery and portal vein circuit inflows for flow monitoring.

3.2.3 Study design

A model of DCD livers was used via the induction of cardiac arrest by a potassium chloride injection (20 mEq; Hospira) at the end of the vascular dissection during the organ-recovery procedure. The donor pigs received in total 30,000 IU of heparin 5 minutes before cardiac arrest. After 45 minutes of cardiac arrest, the organs were flushed with 3 L of cold University of Wisconsin solution (SPS-1, Organ Recovery Systems, Itasca, IL) and stored on ice. During the donor WI time, the blood was collected. Erythrocytes were isolated by soft spinning and were stored in citrate phosphate dextrose adenine (500-mL bag; Terumo, Somerset, NJ).

In the SNEVLP group, the liver was stored for 4 hours on ice (the time frame was designated to simulate the transport time from the donor hospital to the recipient hospital), and this was followed by SNEVLP for 3 hours at 33°C and then CS for 3 hours (10-hour total

preservation time). In the control group, the grafts were continuously stored for 10 hours on ice (**Fig. 8**). At the end of the preservation time, orthotopic pig liver transplantation was performed with an active portojugular shunt (Rotaflow centrifugal pump, Maquet). Two different sets of experiments were performed. First, we performed a nonsurvival study (**n = 5 per group**). In the nonsurvival experiments, the animals were kept alive under anesthesia for 8 hours. Biopsies were obtained during this period, and bile production was measured by cannulation of the common bile duct. The animals were sacrificed at the end of the nonsurvival experiment. The second set of experiments was performed as a survival study (**n = 5 per group**). For the survival study, the animals were kept alive for 7 days. To prevent animal suffering, pigs were sacrificed before the end of the intended survival period in accordance with our animal use protocol and under the supervision of our veterinarian staff if predetermined animal-suffering criteria were met (lethargy, failure to move coordinately, metabolic or respiratory decompensation, and excessive bleeding). At autopsy, the patency of all anastomoses was confirmed. Pigs were exsanguinated while under deep isoflurane anesthesia after central liver and bile duct specimens (each right and left bile duct) had been obtained.

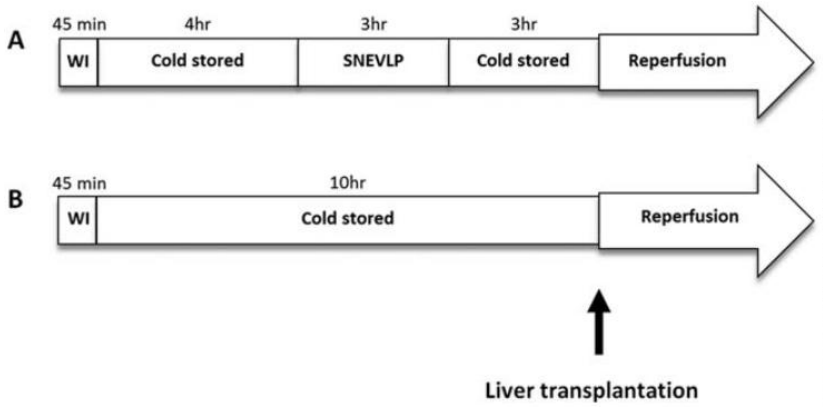

Figure 8. (A) Forty-five minutes of WI was applied to both groups as a model of DCD organ retrieval. Then, the treatment group was exposed to 4 hours of CS plus 3 hours of SNEVLP plus 3 hours of CS. The first CS period was used to simulate transport of the organ to the transplant center, and the second CS period was applied for cooling during recipient hepatectomy and implantation. (B) The control group grafts were convention- ally cold-stored for 10 hours.

b-Galactosidase Assay

b-Galactosidase is a lysosomal enzyme that is rapidly released from Kupffer cells during hepatic reperfusion and is, therefore, considered an early marker of Kupffer cell activa-

tion. (178). We fluorometrically measured b -galactosidase serum levels hourly as described earlier by McGuire et al. (179). b-Galactosidase catalyzes the reaction of the substrate 4-methylumbelliferylgalactoside (MUG) into 4-methylumbelliferone (4-MU), which can be detected fluorometrically by a microplate reader with an excitation wavelength of 340 nm and an emission wavelength of 465 nm. For each well, 10 μL of a 4X diluted serum sample was added to 80 μL of a solution of MUG substrate in a citratephosphate buffer (substrate concentration = 3.33 mmol/L, pH 4.5). Then, the microplate was incubated for 30 minutes at 37°C. The reaction was terminated by the addition of a glycine-NaOH buffer, and this raised the pH above 10. One unit of b-galactosidase is equivalent to 1 nmol of the substrate converted to the product in 1 hour at 37°C. Fluorometric values were compared with a 4-MU standard curve for each reading.

Hyaluronic Acid Assay

Under normal conditions, hyaluronic acid (HA) is metabolized by ECs. We tested its levels to assess EC function. (180) HA was measured with a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). In brief, this assay uses a quantitative sandwich enzyme immunoassay technique. Recombinant human aggrecan, which is precoated in the plate well, binds any hyaluronan of serum and standard samples. After any unbound substances are washed away, enzyme-linked aggrecan is added to the wells. After a wash to remove any unbound aggrecan-enzyme reagent, a substrate solution is added to the wells, and color develops in proportion to the amount of hyaluronan bound in the initial step. The color development is stopped, and the intensity of the color is measured.

Parameters of Hepatocyte and Bile Duct Injury

Aspartate aminotransferase (AST) was measured every hour after blood reperfusion as a marker of hepatocyte injury with a modular bench-top chemistry system (Vitros DT60 II, Ortho Clinical Diagnostics, Rochester, NY). Liver necrosis was assessed by hematoxylin and eosin (H&E) staining at 8 hours after reperfusion. Cleaved caspase-3 staining was used as a marker of apoptosis. The international normalized ratio (INR) and factor V were used as markers of liver function.

Bile production was monitored hourly in the nonsurvival study. Bile fluid was analyzed for lactate dehydrogenase (LDH) content as a marker of bile duct injury. Total serum bilirubin and alkaline phosphatase (ALP) were measured daily in the survival model as a

marker of bile duct damage. Bile duct necrosis was investigated by H&E staining at the end of animal survival.

Histology

At 8 hours after reperfusion in the nonsurvival group and at the end of animal survival at day 7 in the survival group, central liver and bile duct biopsies were taken and stored in 10% formalin overnight and then exchanged for 70% ethanol until paraffin embedding. Sections of 5-µm thickness were cut. Cleaved caspase 3 and CD31 [platelet endothelial cell adhesion molecule (PECAM)] staining were performed via immunohistochemistry. (181) Histology was evaluated by a blinded investigator (H&E) and with image analysis software (cleaved caspase 3, CD31).

For morphometric analysis, stained cells were identified with Spectrum 10.2.2.2317 (Aperio Technologies, Vista, CA). Briefly, slides stained immunohistochemically with cleaved caspase 3 (Cell Signaling Technology, Danvers, MA) and CD31 antibodies (Santa Cruz Biotechnology, Dallas, TX) were scanned at 320 and were qualitatively analyzed with a nuclear (for caspase 3) and cytoplasmic (CD31) positive-pixelcount algorithm.

Statistical Analysis

The data were analyzed with the SPSS 22 statistical package (IBM, Chicago, IL). The Mann-Whitney test was used for the comparison of continuous variables, and the chi-square test was applied for categorical outcomes. The results are presented as means and standard deviations and are considered significant at $P < 0.05$.

3.3 Literature research design

To answer the research question, a systematic literature search was conducted. The doctoral thesis is therefore designed as a review, according to the guidelines of the Cochrane Collaboration. A systematic review is characterized by searching for, selecting, and evaluating all studies that exist on a specific question according to previously defined criteria (182, 183). By identifying suitable studies, research questions should be answered, the state of research should be presented in a science-based manner, and research gaps should be identified. This approach is necessary because several million scientific articles are published every year and it seems hardly possible to read all relevant scientific publications within each discipline. The aim of systematic literature reviews is therefore to identify as many relevant publications on the topic as possible in accordance with the search strategy, to summarize them thematically, to evaluate their level of evidence if necessary, and to record the areas in which research gaps exist to date (184). After the

systematic search has been carried out, the systematic literature analysis takes place, which in this paper will follow the guidelines of the PRISMA protocol, which consists of a checklist consisting of 27 points and a flow chart (Figure 6). The PRISMA statement is generally intended to assist authors in improving the reporting of systematic reviews and meta-analyses (185). After the systematic literature search, duplicates are first removed, then the titles or abstracts of the remaining studies are analyzed ("pre-selected"), excluding inappropriate studies. The remaining titles are then assessed in full text and qualitatively and quantitatively summarized.

3.3.1 Search Strategy

The search procedure for the accomplishment of the present review was divided in three steps. In the first step, a search was performed to obtain further knowledge on the types of liver injury and machine perfusion techniques. The second step involved a search focused on gaining insight on the technical aspects and characteristics of machine perfusion devices. Finally, the third and final step consisted of an extensive database search in order to answer the major research question.

The initial informative search was performed on Google Scholar and involved the following search keywords: liver, machine perfusion, preservation, ex-vivo, transplantation. The search yielded some landmark papers on the field of liver machine perfusion as well as several reviews on the different techniques. Apart from the acquired knowledge and further understanding of the topic, the information collected was used to formulate the final extensive search term which was used in the final stage.

The second phase of the search process involved another manual search on Google Scholar and Web of Science. However, this search was more technical-oriented and the search terms used were: liver, machine perfusion, technology, components, technical aspects, pumps, oxygenators, perfusion circuits. Information on the perfusion device configuration, functional components, technology and engineering was collected. An overview of liver perfusion devices from an engineering and technological perspective is presented in the Machine Perfusion Technology section.

Finally, the third step involved an extensive search on multiple databases. The keywords were derived from the research question and alongside with the information obtained from the initial search, the final search term was determined and formulated. The complete search term that was used was:

('liver transplant' OR 'hepatic transplant*' OR 'liver preservation' OR 'liver graft') AND ('liver perfusion' OR 'machine preservation' OR 'machine perfusion') AND temperature*

AND (perfusate OR 'perfusion solution*' OR 'preservation solution*' OR pulsat* OR nonpulsat* OR 'non-pulsat*' OR 'continuous flow' OR 'continuous perfusion' OR single OR dual) AND [english]/lim*

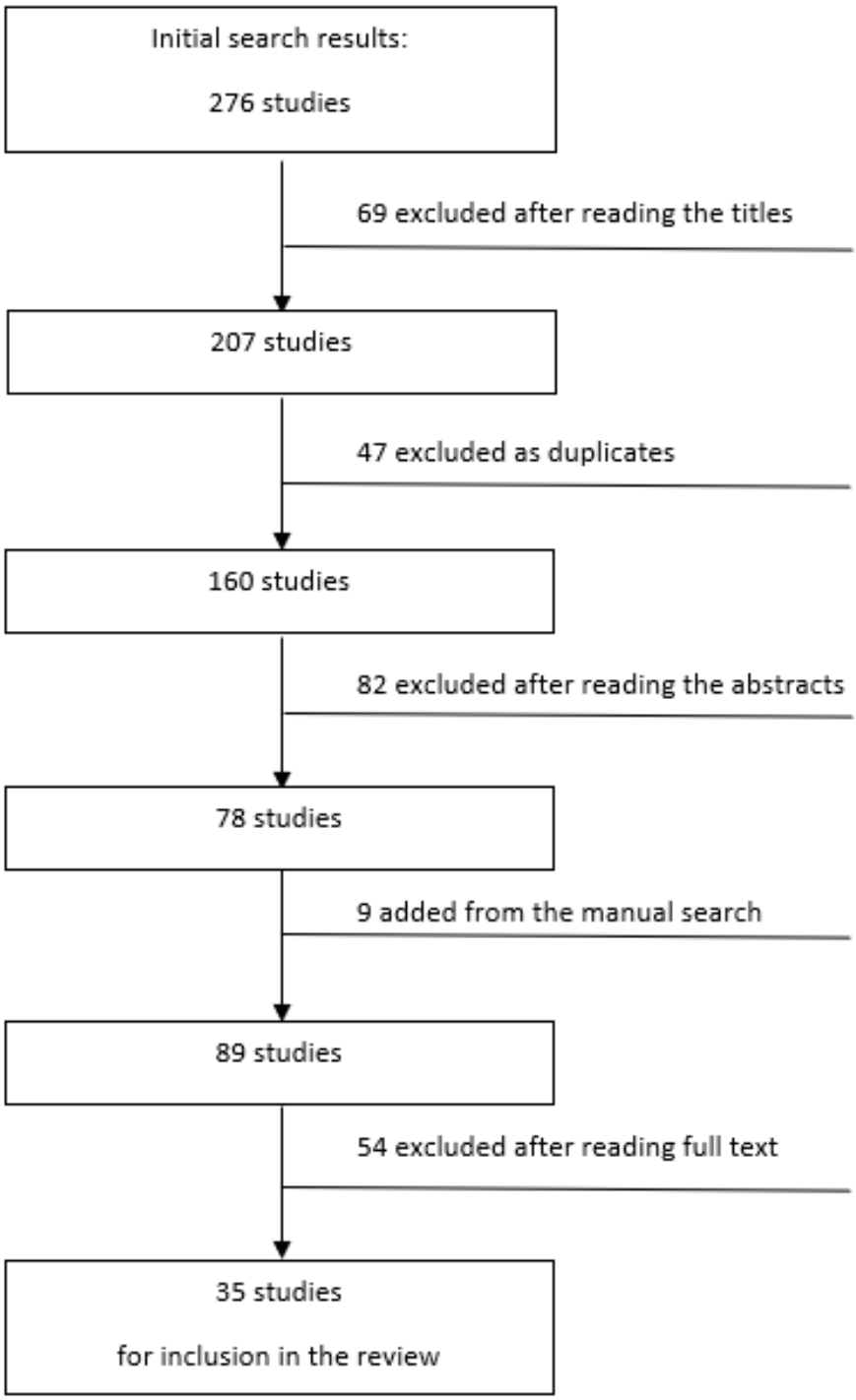

Figure 9. *Flowchart of the systematic literature search process.*

The extensive search was performed on October 23rd 2019, on the following databases: Embase, PubMed and Web of Science. For a study to be included, it had to examine the effect of a machine perfusion technique on the preservation of a human or animal liver graft ex-situ. Abstracts, papers not written in English as well as studies which did not give adequate information on the perfusate, type of flow or perfusion circuit were excluded. Reviews were also excluded from final selection, but were studied to gain more insight on the subject and identify any landmark studies that were not found in the extensive search.

The search yielded 276 results in total, and based on the abovementioned inclusion criteria, 207 were saved after reading only the titles. From those, 47 papers were excluded as duplicates and another 82 were excluded after reading the abstracts (39 reviews, 21 not focusing on MP, 19 not focusing on the preservation outcome and 3 instructional articles). In the remaining 78 papers, 9 informative studies that were found in the initial manual search but not in the systematic database search were added. After reading the full texts of those 87 papers, 35 were selected for inclusion in the literature review. Of the excluded papers, 18 did not provide sufficient information on the examined perfusion parameters, 11 utilized MP for short-term liver resuscitation, 9 utilized in-vivo MP, 13 did not focus on the preservation outcome and in 3 cases the full text was not available. The complete process of the extensive database search is summarized in a flowchart, as depicted in Figure 9. Information from the selected papers was extracted, summarized in Tables 4, 5 and 6 and presented in the Results section.

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4. Results

4.1 Normothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

Hepatocyte injury in DCD livers is reduced by acellular normothermic ex vivo liver perfusion when compared with cold static storage

In a first set of experiments we evaluated graft function and injury during the acellular normothermic perfused preservation (n = 6 per group). Following 60 min warm ischemia and 4 h cold static storage, the livers were perfused for 8 h with 38°C acellular Steen solution. Perfusate ALT levels as a marker of hepatocyte injury during NEVLP remained low with a peak of 55 ± 35 U/L (**Figure 10**). H&E staining on liver biopsies at the end of NEVLP showed minimal necrosis (<5%). Liver synthetic function was preserved during NEVLP as assessed by bile production, oxygen consumption and urea nitrogen (BUN) production. Average rate of bile production during NEVLP was 1.3 ± 1 cc/h, the average oxygen consumption (PO_2 pre liver minus PO_2 post liver) was 430 ± 85 mmHg. Perfusate urea nitrogen levels increased during the perfusion at 0.83 ± 0.2 µmol/L/h.

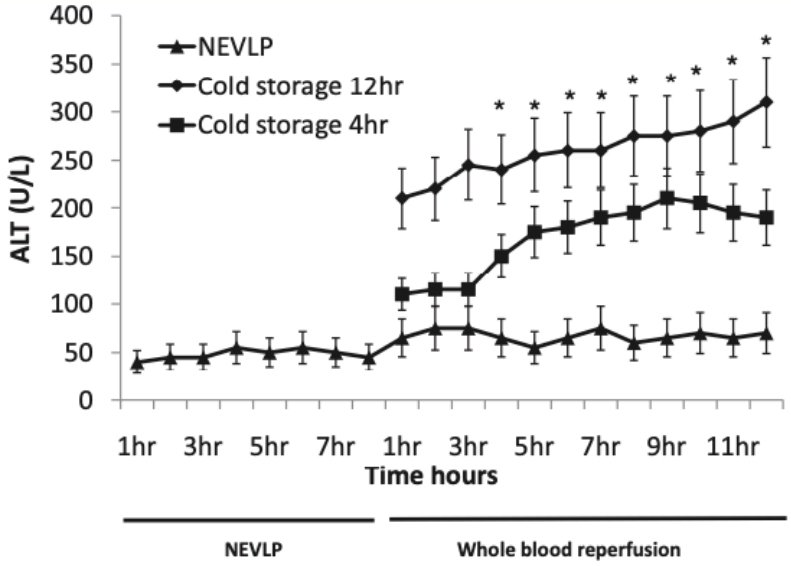

Figure 10: During normothermic perfusion ALT levels remained within the range of normal pig ALT levels (40– 60 U/L). The NEVLP preserved livers had a minimal ALT increase after blood reperfusion. In contrast, cold static preserved livers had a five- to six-fold increase of ALT compared with the NEVLP group (n = 6, ANOVA, *p < 0.001 at each time point 4 or 12 h cold vs. NEVLP).

Next, we compared liver injury and function in normothermic and cold preserved livers after ex vivo blood reperfusion. DCD livers had decreased mean ALT levels after NEVLP compared to grafts with either short (A) (55 ± 35 U/L vs. 163 ± 85 U/L; p = 0.03) or prolonged (B) (55 ± 35 U/L vs. 350 ± 125 U/L; p < 0.001) cold storage.

H&E staining of short (A) and prolonged (B) cold static preserved livers revealed 45% (A) and 55% (B) hepatocyte necrosis at the end of ex vivo blood reperfusion respectively, whereas hepatocyte necrosis was <10% in the normothermic preserved grafts (p = 0.01; **Figure 11A–C**). The mean oxygen extraction in the liver after 12 h ex vivo blood reperfusion in the NEVLP group was 410 ± 58 mmHg. In contrast, DCD grafts with 4 and 12 h cold static preservation had a rapid drop in oxygen consumption after blood reperfusion indicating a deteriorating metabolic activity (**Figure 12**) with an oxygen extraction of only 250 ± 65 mmHg (A) and 200 95 mmHg (B) after 12 h of storage respectively (p = 0.015).

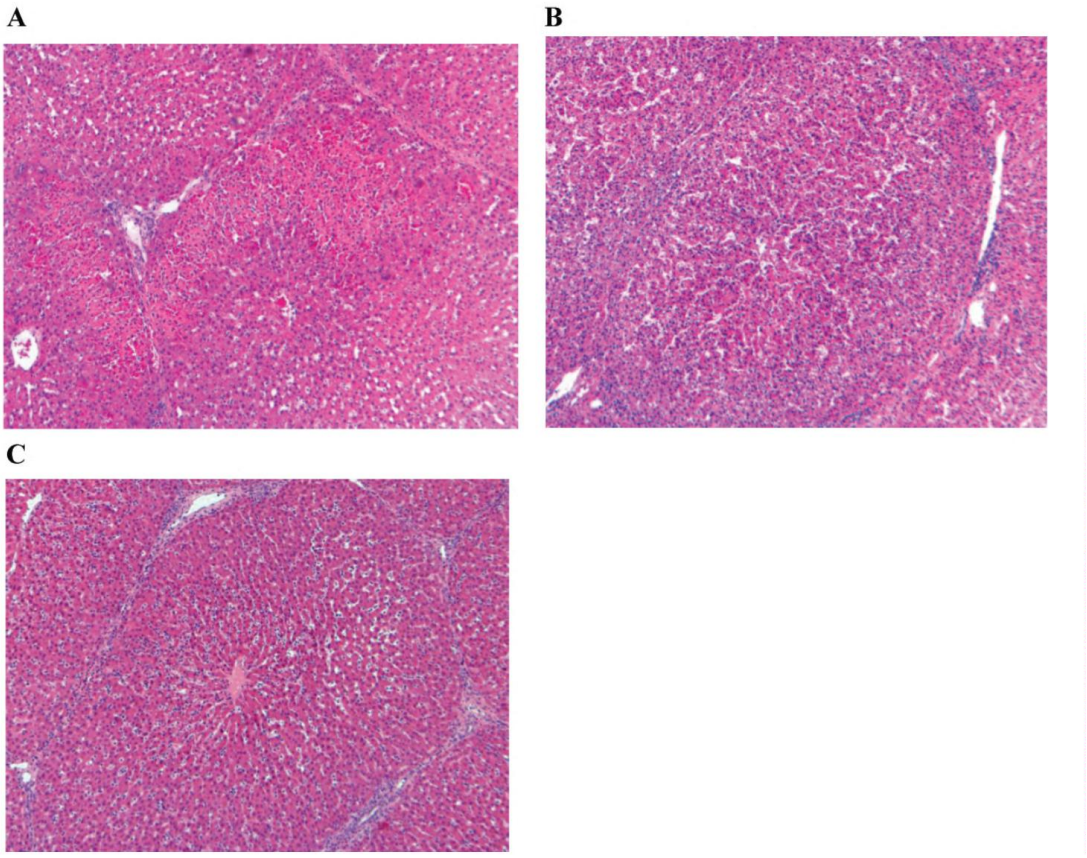

Figure 11: DCD liver grafts were preserved either by 4 h (A) or 12 h (B) cold static storage or 4 h cold storage plus 8 h NEVLP (C). After organ preservation all grafts were reperfused for 12 h with diluted blood. H&E staining was performed on biopsies obtained at the end of blood reperfusion. Short (A) and prolonged (B) cold static stored DCD grafts had 45% and 50% necrosis of the liver tissue respectively, while minimal

necrosis (<10%) was present in livers which were preserved by normothermic perfusion (C).

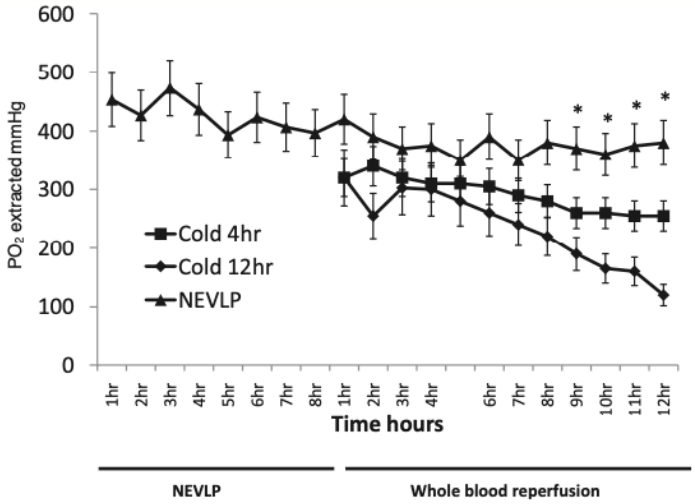

Figure 12: Oxygen extraction of DCD livers after 4 or 12 h cold static storage or 4 h cold storage plus 8 h NEVLP. Oxygen extraction remained stable in normothermic preserved livers after blood reperfusion as a marker of maintained metabolism. Cold static preserved DCD grafts had a declining oxygen extraction indicating deteriorating metabolic activity (n = 6, *p < 0.05, 4 and 12 h cold stored vs. NEVLP, ANOVA).

Normothermic perfused preservation improves HA perfusion in DCD liver grafts

Reduced arterial blood flow has been proposed by others as a potential mechanism of ITBS in DCD grafts (1). Therefore, we investigated hepatic arterial liver perfusion with CT angiography in the DCD liver grafts after either short (A) or prolonged (B) cold static storage or normothermic perfusion (C) followed by 12 h ex vivo whole blood reperfusion (n = 6 per group). HA perfusion of the peripheral liver parenchyma was preserved in DCD livers with normothermic perfused preservation (355 ± 70 HU). In contrast, DCD liver grafts with either short or prolonged cold static storage had a significant loss of peripheral HA perfusion (190 ± 8 HU and 170 ± 5 HU; **Figure 13A–C**). In addition, during the last 4 h of blood reperfusion the mean arterial flow in short (A) and prolonged (B) cold stored graft was significantly lower, than NEVLP (C) preserved livers (180 ± 35 cc/min A; 120 cc/min B; vs. 340 ± 85 cc/min C).

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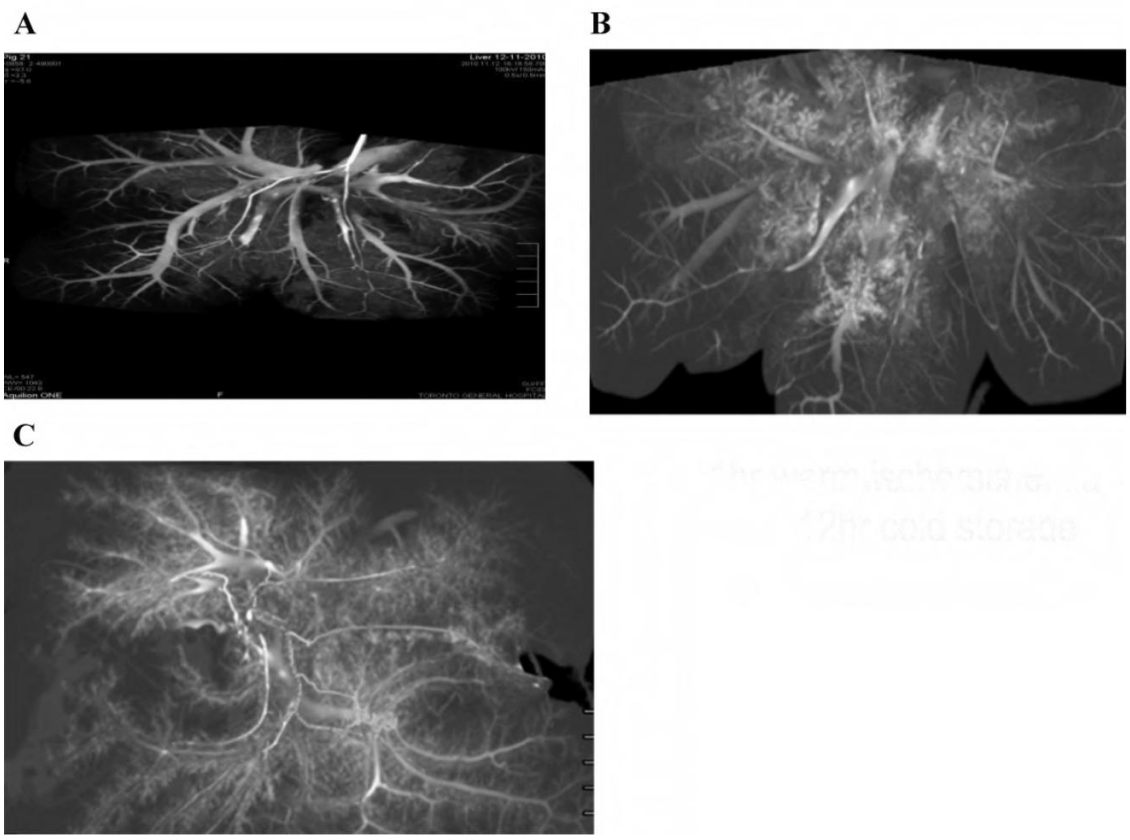

Figure 13: Hepatic artery CT angiography of DCD livers 12 h after ex vivo blood reperfusion. The livers were preserved by either 4 h (A) or 12 h (B) cold static storage, or 4 h cold static storage plus 8 h NEVLP (C). Cold static preserved DCD grafts had a significant loss of arterial perfusion of the liver tissue. In contrast, arterial blood flow was preserved in DCD livers with normothermic perfused preservation.

Normothermic perfused preservation improves bile duct function and reduces bile duct injury of DCD grafts

Bile flow was assessed after short (A) and prolonged (B) cold static storage versus normothermic perfused pre- served liver grafts (C) after ex vivo whole blood reperfusion. No differences were observed between bile flow in all three groups (n = 6 per group) (1.5 ± 0.6 cc/h [A]; 1.3 ± 0.8 cc/h [B] vs. 1.8 ± 1.1 cc/h; p = 0.5). Next, we investigated bilirubin, total bile acids and total phospholipid content in the bile of the three groups as a marker of bile function. The bilirubin concentration was significantly higher in the bile of grafts after normothermic perfused preservation versus short (67 ± 12 µmol/L vs. 18±4 µmol/L; p = 0.01) or prolonged (67 ± 12 µmol/L vs. 10±2 µmol/L; p < 0.001) cold storage. Similarly, bile acids were also significantly higher in normothermic perfused grafts versus short (83 762 ± 4530 µmol/L vs. 2135 ± 820 µmol/L; p = 0.015) or prolonged (83762 ± 4530 µmol/L vs. 1550 ± 550 µmol/L; p < 0.001) cold static preserved livers.

Finally, after blood reperfusion bile phospholipid concentration was higher in DCD grafts with normothermic perfusion versus short (343 ± 23 µmol/L vs. 102 ± 22 µmol/L; p = 0.01) or prolonged (343 ± 23 µmol/L vs. 62 8 µmol/L; p < 0.001) cold storage (**Table 2**).

Bile component	NEVLP	Cold storage 4h	Cold storage 12h	p-value
Bilirubin (µmol/L)	A: 67 ± 12	B: 18 ± 4	C: 10 ± 2	A vs. B = 0.01 A vs. C < 0.001
Phospholipids (µmol/L)	A: 343 ± 23	B: 2135 ± 82	C: 62 ± 8	A vs. B = 0.01 A vs. C < 0.001
Bile acids (µmol/L)	A: 83762 ± 4530	B: 21235 ± 82	C: 1550 ± 550	A vs. B = 0.015 A vs. C < 0.001
LDH	A: 522 ± 83	B: 1830 ± 625	C: 2690 ± 823	A vs. B = 0.015 A vs. C < 0.001

Table 2: Bile components after reperfusion. Bile content of DCD grafts after cold static preservation or combined 4 h cold preservation and 8 h acellular normothermic perfusion. Bile samples were obtained after 6 h blood reperfusion. Bile of DCD grafts with normothermic preservation had fivefold increased bilirubin, phospholipid and bile acid content when compared with cold static preserved livers. In contrast, LDH in bile as a marker of cell death was fivefold increased in livers after cold static preservation (n = 6, ANOVA).

Bile LDH was determined as a marker of cell death within the biliary tract (Table 1) (12). LDH levels were significantly decreased in the bile analyzed from normothermic perfused grafts in comparison to either short (522 ± 83 µmol/L vs. 1830 ± 625 µmol/L; p = 0.015) or prolonged (522 ± 83 µmol/L vs. 2690 ± 823 µmol/L; p < 0.001) cold storage. Finally, H&E staining of bile ducts was performed. DCD organ retrieval and cold storage alone without reperfusion did not result in bile duct necrosis. In contrast, following 12 h of blood reperfusion all DCD liver grafts with short or prolonged cold static storage had 100% necrosis of the bile duct mucosa with no viable bile duct mucosa. Four out of five DCD grafts with NEVLP preservation had no detectable bile duct injury with intact mucosa lining of the bile duct and the end of blood reperfusion. One liver demonstrated partial bile duct injury (30%, p = 0.001; **Figure 14A–D**).

Normothermic ex vivo perfusion decreases liver injury in DCD grafts after liver transplantation

Livers were exposed to 60 min of cardiac arrest as described above. The NEVLP group was exposed to 4 h cold storage and 4 h normothermic perfusion as described above. In comparison, the control group was preserved with 8 h cold static storage only. At the end of preservation period, orthotopic liver transplantation was performed and serum AST

was measured hourly for 8 h as a marker of liver injury. Mean serum AST levels were significantly higher in animals receiving a cold stored versus NEVLP preserved DCDliver (1809 ± 205U/L vs. 524 ± 187U/L, n = 6 per group; **Figure 15**). Bile production was monitored via a catheter inserted in the common bile duct. No difference in bile volume was observed between cold stored and NEVLP groups after transplantation (mean 2.5 ± 1.2 cc/h vs. 2.8 ± 1.4 cc/h; p = 0.2).

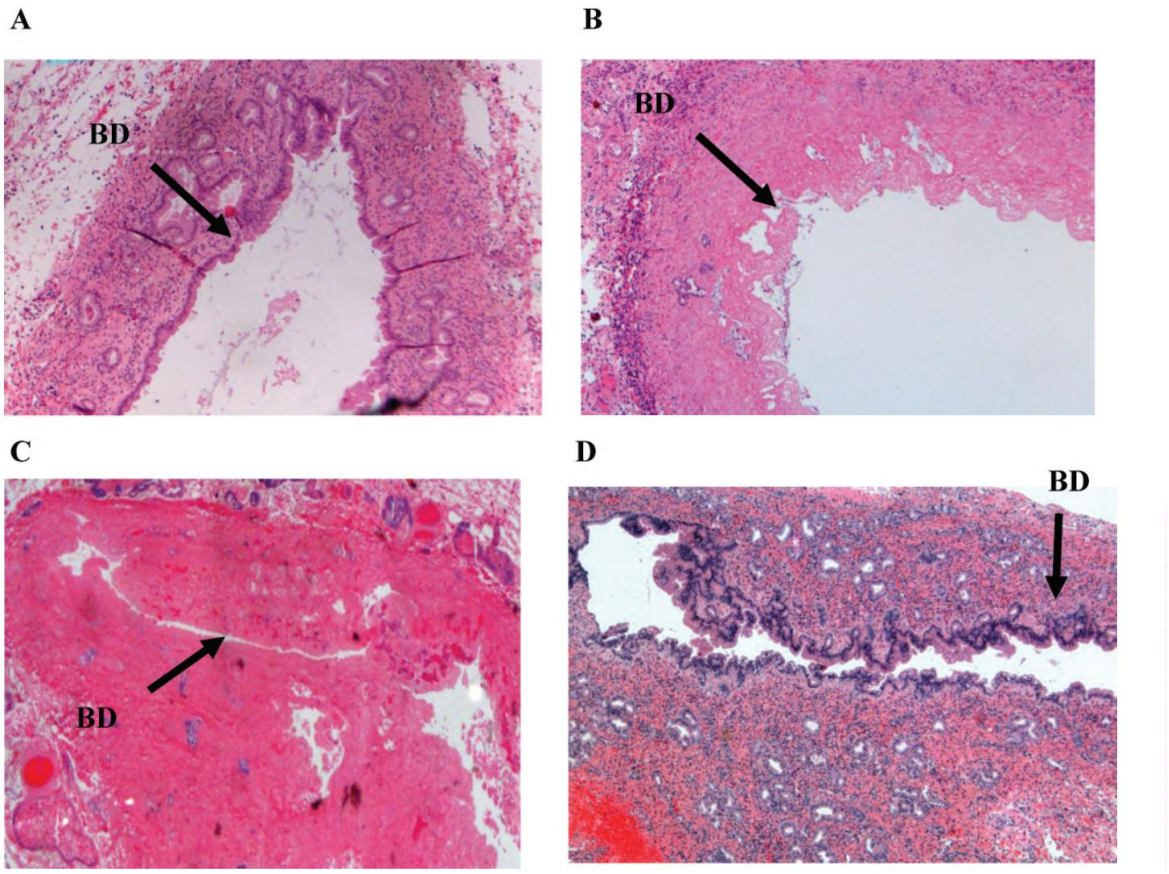

Figure 14: H&E staining of bile ducts from DCD liver grafts. Without reperfusion no bile duct injury was detectable despite 1 h warm plus 4 h cold ischemia (A). In contrast, DCD liver grafts which were preserved for either 4 h (B) or 12 h (C) cold storage had complete bile duct necrosis after 12 h blood reperfusion. NEVLP preservation of DCD grafts prevented bile duct necrosis at the end of 12 h blood reperfusion (D) (BD, bile duct).

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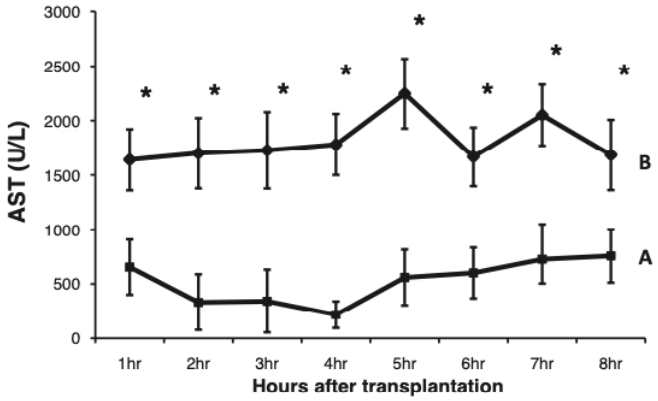

Figure 15: AST as a marker of liver injury following orthotopic pig liver transplantation using DCD grafts. Group A received a DCD liver graft with combined cold storage (4 h) and NEVLP (4 h), while in group B the DCD graft treated with 8 h cold storage only. AST was significantly reduced with NEVLP preservation (n = 6, *p < 0.05, Student's t-test).

4.2 Subnormothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

Liver Injury in SNEVLP and CS DCD Liver Grafts After Transplantation

First, we determined whether SNEVLP alone induced injury in DCD liver grafts. The liver grafts were exposed to 45 minutes of WI plus 4 hours of CS before SNEVLP. AST as a marker of hepatocyte injury did not increase during 3 hours of SNEVLP with a mean perfusate AST level of 152 ± 23 U/L after 1 hour and 146 ± 25 U/L after 3 hours (P = 0.8). H&E staining of liver tissues at the end of SNEVLP preservation showed minimal liver necrosis (<5%). This minimal necrosis during ex vivo perfusion demonstrated that our perfusion system did not induce liver damage by itself.

Next, we compared liver injury in SNEVLP-preserved and cold-preserved livers after orthotopic liver transplantation. Serum AST levels at 2 hours after transplantation were significantly lower in SNEVLP grafts versus CS grafts (261 ± 175 versus 691 ± 261 U/L, P = 0.008; **Fig. 16**). However, peak AST levels within 24 hours after reperfusion were similar between SNEVLP and CS grafts (3198 ± 826 versus 2585 ± 1102 U/L, P = 0.46). After 8 hours of reperfusion, SNEVLPpreserved grafts versus cold-preserved DCD grafts had a trend toward reduced cleaved caspase 3 staining (0.7% ± 0.6% versus 3.3% ± 1.9% positive cells, P = 0.07) as a marker of apoptosis (**Fig. 17**).

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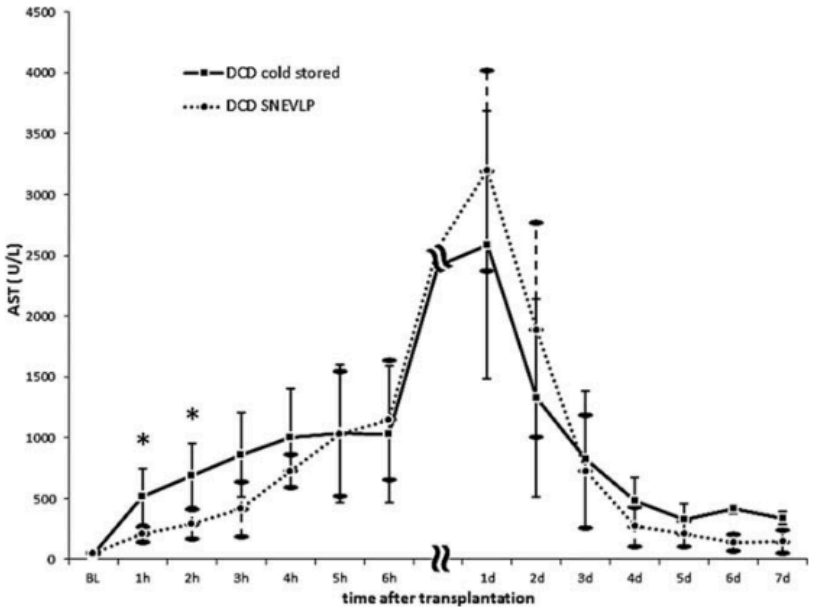

Figure 16. AST after DCD liver transplantation with CS- and SNEVLP-preserved grafts. AST levels were decreased early after transplantation in the SNEVLP group, but the 2 groups reached similar peak values within 24 hours after transplantation (n = 5 for each group, *P < 0.05).

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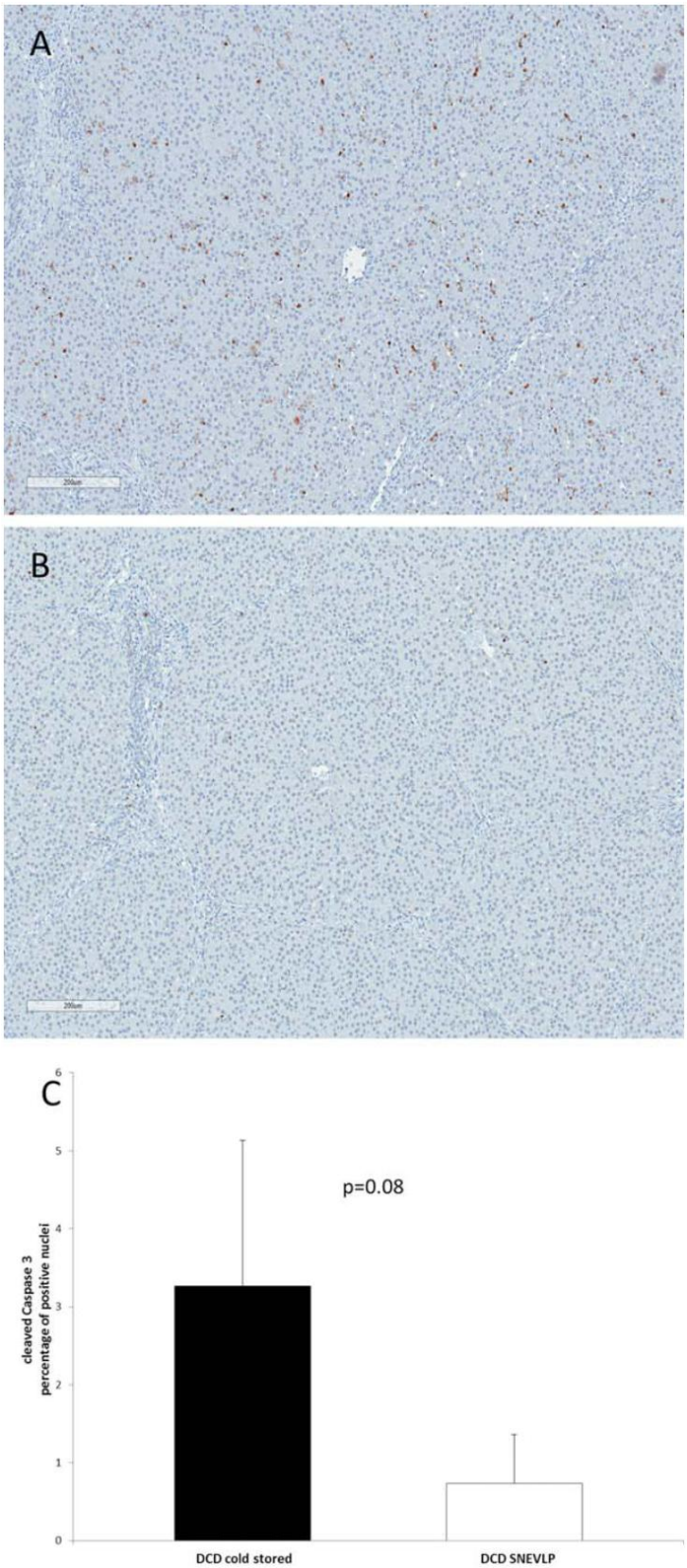

Figure 17. Cleaved caspase 3-positive cells were evaluated with image analysis software in scanned slides of liver tissue obtained 8 hours after reperfusion. (A) Immunohistochemistry for cleaved caspase 3 in the CS group. (B) Immunohistochemistry for cleaved caspase 3 in the SNEVLP group. (C) CS

DCD grafts had a trend toward more caspase 3 staining than SNEVLP-treated liver grafts (n = 5 for each group, P = 0.07).

b-Galactosidase, a marker of Kupffer cell activation, was determined after transplantation. As shown in **Fig. 18**, serum levels of b-galactosidase were significantly lower in SNEVLP grafts versus CS grafts between 2 and 6 hours after reperfusion, and this indicated reduced Kupffer cell activation with SNEVLP preservation.

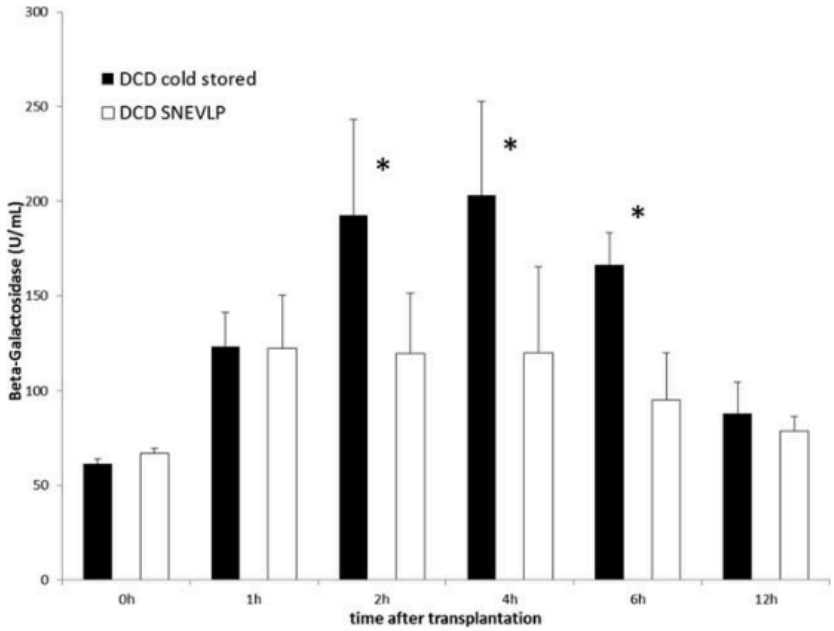

Figure 18. b-Galactosidase serum levels after DCD liver transplantation as a marker of Kupffer cell activation. b-Galactosidase levels were decreased in pigs receiving an SNEVLP graft versus CS DCD graft in the reperfusion phase between 2 and 6 hours (n = 5 for each group, *P < 0.05).

SNEVLP Instead of CS Reduces EC Injury in DCD Grafts

To assess EC viability, we stained liver tissue via CD31 immunohistochemistry at 8 hours after liver transplantation. Slides were analyzed with image analysis software. SNEVLP-preserved DCD livers had intact sinusoidal EC lining and minimal EC injury ($7.3 \times 10^{-4} \pm 2.6 \times 10^{-4}$ cells/lm^2). In contrast, CS DCD grafts had lost the sinusoidal EC lining, with only clumps of EC remaining; this indicated severe EC injury ($3.7 \times 10^{-4} \pm 1.3 \times 10^{-4}$ cells/lm^2, P = 0.03; **Fig. 19**).

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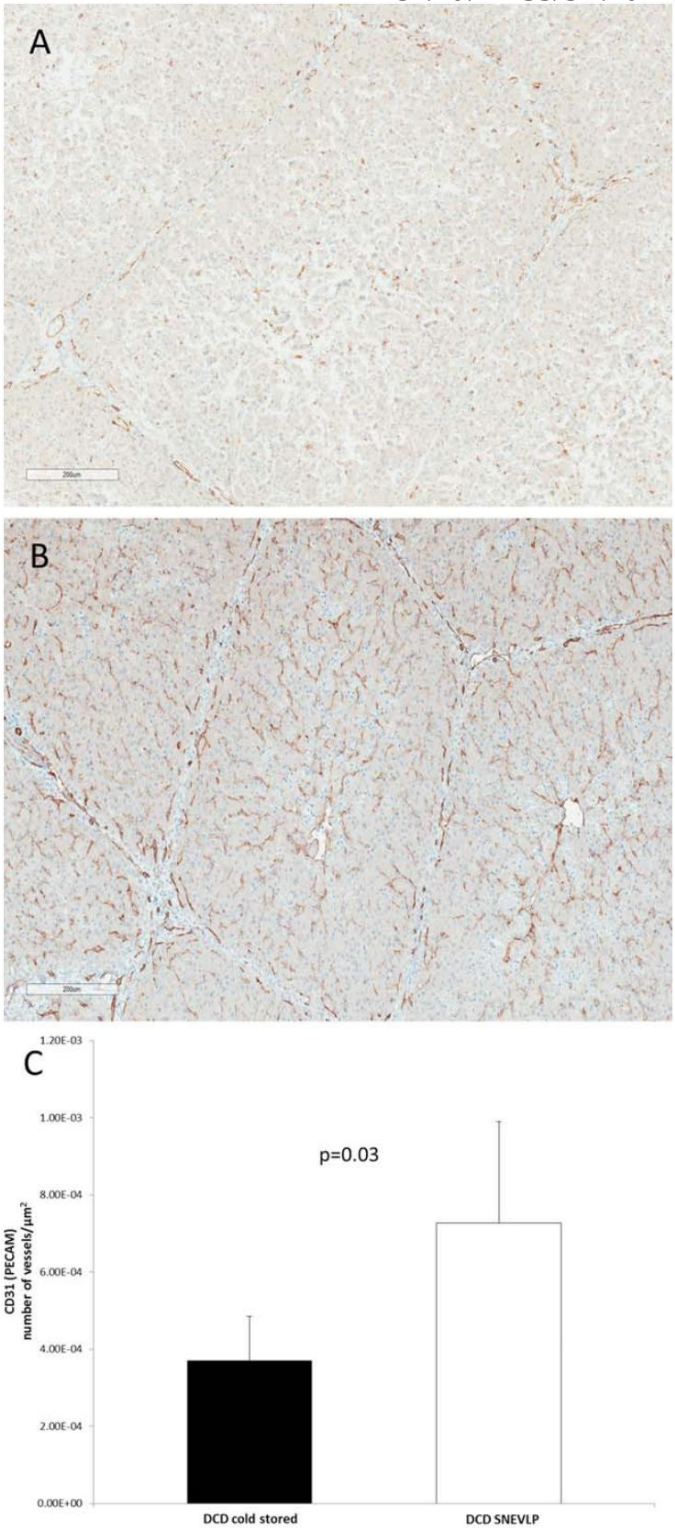

Figure 19. CD31 (PECAM) immunohistochemistry to assess sinusoidal EC injury. (A) DCD grafts in the CS group had severe injury of sinusoidal ECs 8 hours after transplantation. (B) SNEVLP-treated DCD grafts had preserved CD31 staining, which indicated reduced EC injury. (C) CS DCD grafts had a signifi- cantly lower vessel density as a marker of EC injury than SNEVLP grafts as assessed with imaging analysis software (n = 5 for each group, P = 0.03).

HA serum levels were assessed after liver transplantation as a marker of EC function. HA is cleared by ECs, and increased HA levels correspond to decreased EC function. HA serum levels increased continuously after transplantation in CS grafts, whereas HA levels remained stable in SNEVLP DCD grafts. At 3 hours after transplantation, HA serum levels were significantly reduced in SNEVLP livers versus CS livers (1077 ± 711 versus 2476 ± 364 ng/mL, P = 0.01), and this indicated improved EC function in SNEVLP DCD grafts (**Fig. 20**).

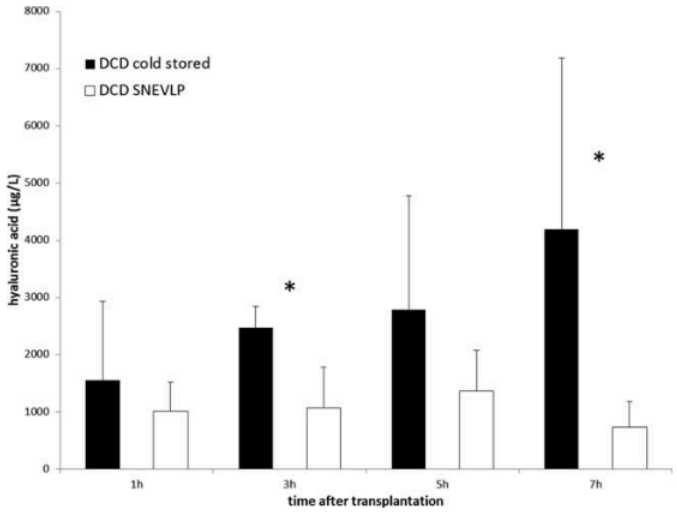

Figure 20. HA serum levels were evaluated after transplantation in CS- and SNEVLP-treated groups as a marker of HA clearance by ECs. Animals receiving a CS DCD liver graft had increased HA levels in comparison with pigs receiving a DCD graft preserved with the SNEVLP protocol (n = 5 for each group, *P < 0.05).

SNEVLP Instead of CS Reduces Bile Duct Injury and Improves Biliary Function After DCD Liver Transplantation

Hourly bile flow during the first 8 hours after transplantation was similar in SNEVLP and CS livers (**Fig. 21A**). LDH was measured in bile fluid as a marker of biliary epithelial injury. SNEVLP versus CS livers had significantly lower bile LDH levels 3 hours after transplantation (14 ± 10 versus 60 ± 18 lmol/L, P = 0.02; **Fig. 21B**).

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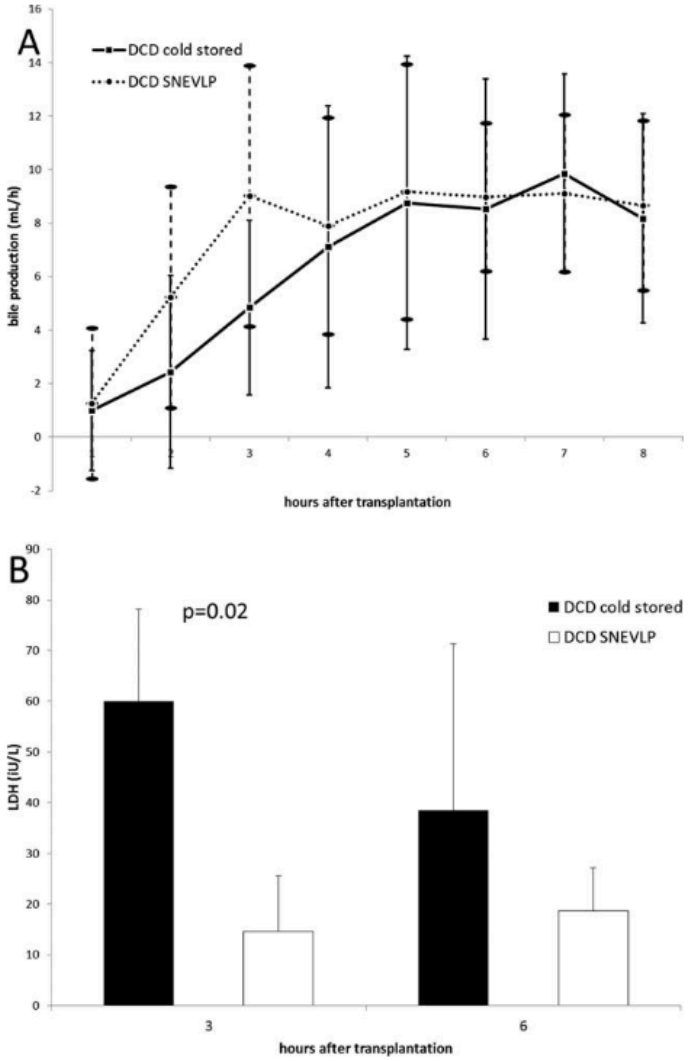

Figure 21. Hourly bile flow after transplantation (A) CS-preserved and SNEVLP-treated liver grafts demonstrated similar bile production 4 hours after reperfusion (n = 5 for each group). (B) The bile LDH content at 3 hours after reperfusion as a marker of biliary injury was significantly lower in the SNEVLP grafts versus the CS grafts (n= 5 for each group, P = 0.02).

Bile duct necrosis was investigated via H&E staining 7 days after transplantation or at the end of animal survival. Severe bile duct necrosis was present in 3 of 5 CS grafts. In contrast, no bile duct necrosis was observed in SNEVLP DCD grafts (P = 0.03; **Fig. 22**). Bile duct necrosis was associated with increased ALP levels after transplantation (**Table 3**).

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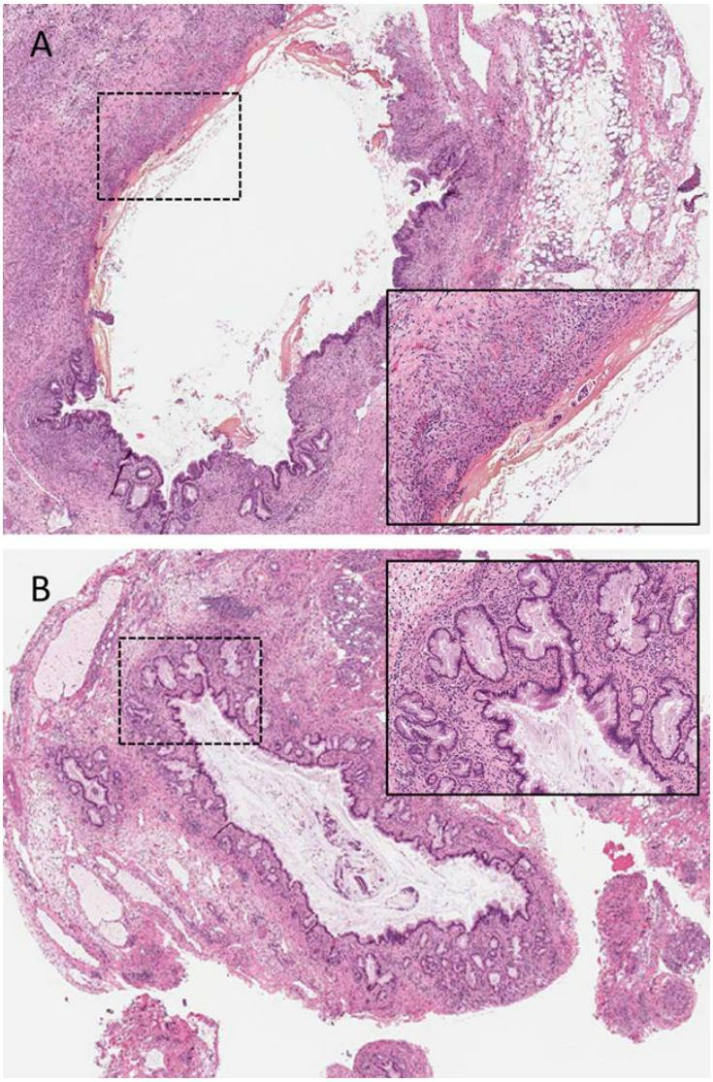

Figure 22. H&E staining of bile ducts in the CS and SNEVLP groups. (A) Massive necrosis in the CS livers. (B) The bile duct mucosa was completely preserved in all SNEVLP DCD grafts.

	Pigs With Bile Duct Necrosis (n = 3)	Pigs Without Bile Duct Necrosis (n = 7)	*P* Value
Total bilirubin peak (μmol/L)	32 ± 30	15 ± 7	0.58
ALP peak (U/L)	369 ± 45	203 ± 80	0.03

Table 3. ALP and Bilirubin as Markers of Bile Duct Injury in Pigs With and Without Biliary Necrosis

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Serum bilirubin and ALP levels were measured at various time points after liver transplantation as markers of bile duct injury. As shown in **Fig. 23A,** serum bilirubin levels were lower in animals receiving an SNEVLP graft versus a CS graft, but the probability was not significant. Similarly, serum ALP levels were lower in pigs receiving DCD grafts preserved with SNEVLP versus CS; in this case, there was significant probability for postoperative days 3 and 4 (**Fig. 23B**).

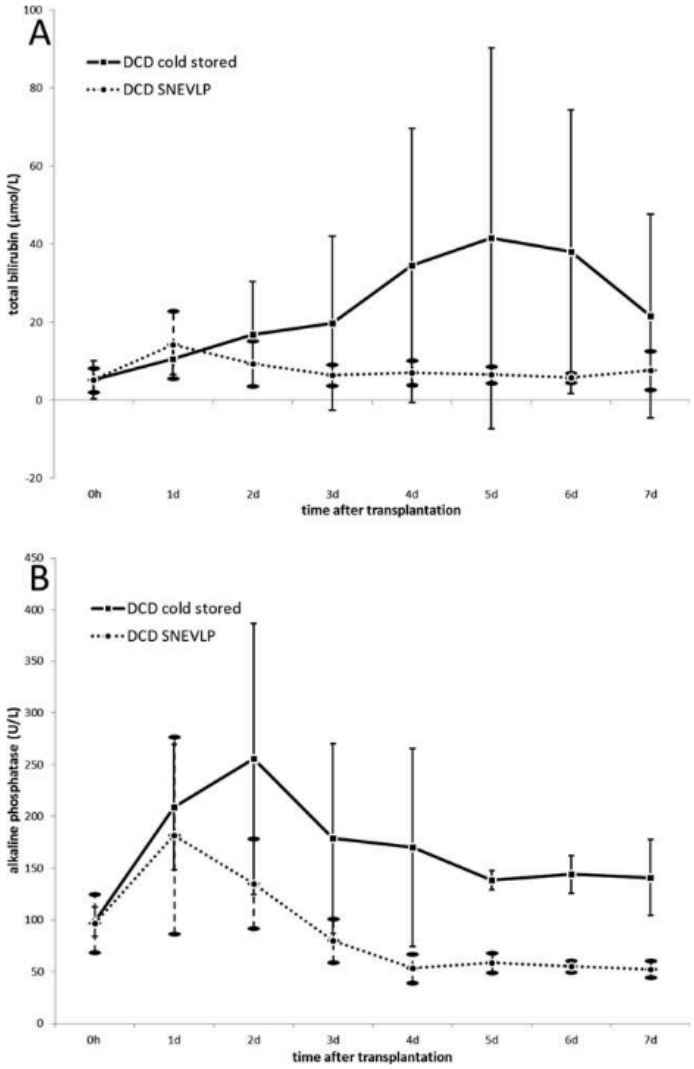

Figure 23. (A) Total bilirubin levels after DCD liver transplantation. Pigs in the SNEVLP group had uniformly low bilirubin levels in a physiological range after transplantation; bilirubin levels in pigs receiving a CS DCD liver graft were higher and more heterogeneous, but there was no significant difference from the SNEVLP group (n = 5 for each group). (B) ALP after DCD liver transplantation. Pigs in the SNEVLP group had lower ALP levels after transplantation in comparison with pigs receiving a CS DCD liver graft (n = 5 for each group, $P < 0.05$).

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Liver Function and Animal Survival After Liver Transplantation

Animal survival was considered permanent after 7 days after transplantation. Three animals with CS grafts died before the end of the survival period: 1 after 12 hours and 2 on day 5 after liver transplantation. In contrast, 2 deaths were registered in the SNEVLP group: the first on day 5 after transplantation and the second on day 7 (log-rank test, P = 0.13; **Fig. 24**). No significant difference between the 2 groups was observed in the coagulation parameters (INR ratio or factor V levels; **Fig. 25**). INR was normalized within 48 hours of transplantation in both groups.

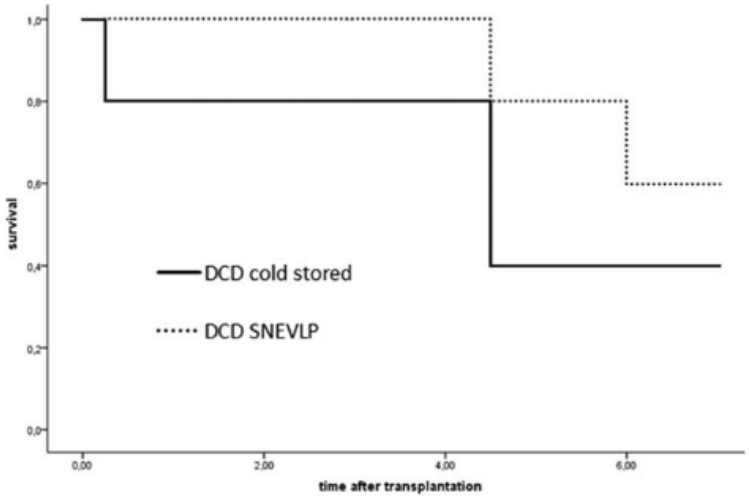

Figure 24. Survival curve after DCD liver transplantation in the CS and SNEVLP groups. Pigs receiving a CS graft had lower but not significantly different survival in comparison with pigs treated receiving an SNEVLP graft (n = 5 for each group, P = 0.13).

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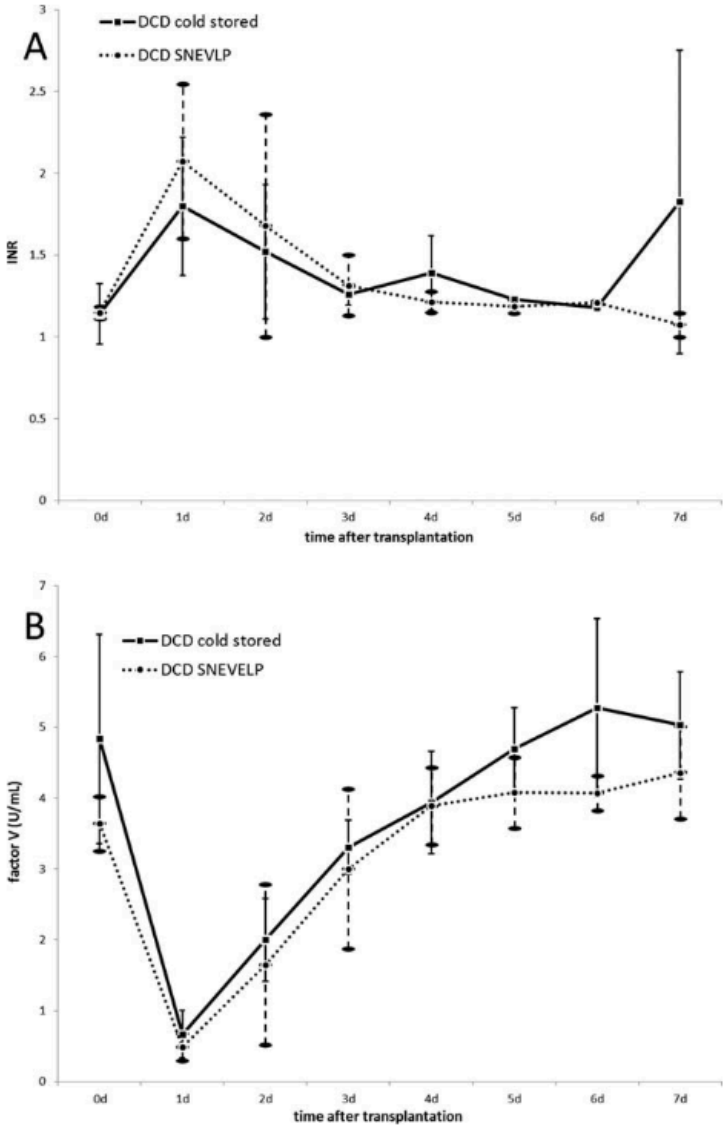

Figure 25. (A) INR and (B) factor V as parameters of liver function at the baseline and after DCD liver transplantation in pigs receiving either a CS graft or a graft treated with SNEVLP. Liver function was similar in the 2 groups (n = 5 for each P = *not significant* for all time points).

4.3 Literature search results

4.3.1 Hypothermic Machine Perfusion

As depicted in **Table 4**, hypothermic machine perfusion (HMP) has been shown by several research groups to be a safe and feasible liver preservation approach. Several research groups have performed HMP via the PV only, however there are several experimental setups that established dual perfusion, via PV and HA. The predominant type of flow in HMP is continuous and is used in almost every single perfusion setup as well as in several dual circuits. Nevertheless, van der Plaats et al. and van der Rijn et al. have created dual HMP settings in which the HA is perfused in a pulsatile way, while perfusion via the PV is continuous. Regarding perfusates, it is observed that both oxygenated and non-oxygenated media are utilized by different experimental systems. The perfusate that is most widely used is the University of Wisconsin (UW) MP solution, which is typically oxygenated. In addition, Vasosol has also been used by Guarrera et al. (121, 142), however in an non-oxygenated manner. Alternatively, oxygenated Lifor and Belzer solutions have also been utilized in the studies included in this review.

Generally, it is obvious that -in all cases- HMP leads to lower levels of AST, ALT and LDH, high ATP and factor V levels as well as reduced histological damage in the liver, especially when compared to static cold storage. Continuous and pulsatile flow modes are directly compared by Dutkowski et al. (88), who showed that pulsatile flow was associated with higher ATP levels and energy charge. Regarding graft viability in the recipient, 2 non-oxygenated HMP studies by Guarrera et al. reported 1 case of early allograft dysfunction (EAD) as well as 1 incident of primary nonfunction (PNF) alongside with 6 cases of EAD respectively. On the other hand, oxygenated HMP yielded no incidents of PNF according to Dutkowski et al., while van Rijn et al. reported 100% 6-month graft survival when HMP with an oxygenated medium was used. (126)

Notably, the application of HMP is either continuous or end-ischemic, where cold ischemia time (CIT) can last for several hours. In addition, the duration of the perfusion may vary from 1-2h up to more than 10h, depending on the experimental setting. It is evident that the perfusion duration of HMP is generally limited. Dutkowski et al. (88) and van der Plaats et al. (118), who performed long-term preservation of the liver for 10 and 24 hours respectively, used dual perfusion with pulsatile HA flow and continuous PV flow and utilized oxygenated UW solution as perfusate. On the other hand, Dirkes et al. (186) performed HMP for an extended period of 20 hours, via PV only and with pulsatile

flow, using oxygenated Belzer solution as the perfusate. It is observed that non-oxygenated HMP is used for limited preservation times, alongside with most single perfusion setups.

4.3.2 Normothermic Machine Perfusion

It is evident from **Table 5** that NMP is a widely used MP technique that is mainly compared with the golden standard of CS in the literature. In all cases, the comparison indicates that livers preserved with NMP displayed lower levels of AST, ALT and LDH, less histological damage and no or insignificant necrosis compared to CS (71, 92, 120, 153, 187). Furthermore, biliary tree and hepatocyte integrity as well as bile production and composition were maintained during perfusion. (117, 120, 123, 187, 188).

All NMP setups in literature apply dual perfusion HA and PV, while also utilizing oxygenated perfusion media. There is no direct comparison between different flow modes, but in most setups continuous flow is implemented and only Dirkes et al. used single perfusion with pulsatile flow (186). Nevertheless, there have been several dual-circuit systems which have applied continuous PV and pulsatile HA flow (117, 120, 153, 189). It is observed that cellular perfusates are used, mainly full blood or packed red blood cells (RBCs) dissolved in a solution such as Steen solution, Gerofundin, William's Medium E or Gelofusine. Boehnert et al. performed NMP with an acellular medium, using oxygenated Steen solution (187). Schlegel et al. compared the effect of full blood and leukocyte- and platelet-depleted blood and yielded similar results in terms of liver viability, while also suggested that bile flow during perfusion is independent of the use of any of the two perfusates (189).

The literature shows that NMP is mainly used for longer perfusion durations, as many studies apply it 10 hours or more (120, 169, 187), while others investigated the outcome of NMP for 24 hours (92, 123, 190). Blood was used as the perfusate in all of these setups, which achieved successful long-term liver preservation with low AST, ALT and LDH levels, insignificant histological damage and necrosis. Of note, Butler et al. accomplished successful 72h liver preservation with NMP, using a dual-perfusion system with continuous flow and donor blood as perfusate (95).

4.3.3 Subnormothermic Machine Perfusion

The literature search results for subnormothermic machine perfusion (SNMP) and demonstrated in **Table 6**. Livers preserved with SNMP exhibit lower AST, ALT and LDH levels than static cold storage (SCS), higher ATP and energy levels as well as more bile production compared to those that underwent CS (97, 99, 146, 191-193). In addition, SNMP led to normal hepatocyte morphology, no sinusoidal epithelium injury or necrosis and less IRI than SCS (96, 191). Vairetti et al. compared SNMP to HMP and concluded that HMP led to lower AST and ALT levels, whereas SNMP allowed higher ATP levels and greater bile flow (99). Also, SNMP reduced the release of AST, ALT and GGT into the bile, compared to static cold storage.

Oxygenated perfusates were utilized in all the included papers. The type of solution varies between different research groups and perfusates such as Krebs-Henseleit, University of Wisconsin (UW), William's medium E, Celsior and acellular hemoglobin-based oxygen-carrying (HBOC) solution are used. Perfusion is performed either via the PV only or via both the PV and HA, with continuous flow being the predominant type of flow. Fontes et al. performed dual SNMP with pulsatile flow in the HA and continuous in the PV (191). The results showed much lower AST and ALT levels as well as significantly higher bile production compared to static cold storage.

None of the included papers investigated long-term liver perfusion with SNMP. SNMP was mainly applied for around 6 hours, with several research groups employing 3h-perfusion models. Fontes et al. displayed the longest preservation duration (mean 7.47 hours) and achieved successful SNMP with a dual circuit, continuous flow in the PV and pulsatile in the HA. The group used acellular and oxygenated HBOC solution as perfusate (191).

5. Discussion

5.1 Normothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

In a pig model that mimics DCD liver transplantation, we demonstrated that normothermic liver perfusion with an acellular perfusion solution resulted in improved arterial perfusion, decreased hepatocytes injury and reduced markers of bile duct injury. The current technology for normothermic perfusion requires equipment that is difficult to transport and run under mobile conditions. Thus, for the foreseeable future liver grafts will likely be flushed at the donor center with a cold preservation solution and then transported back to the transplant center for normothermic perfusion and storage. These steps will result in a cold ischemia time of approximately 3–5 h before normothermic perfusion can be initiated. NEVLP has been previously studied in large animal models by several groups using either whole blood or diluted blood as perfusate; however, none of these studies have combined NEVLP with cold storage as it would be used in clinical practice. Butler et al. (95) demonstrated that NEVLP using whole blood can be performed up to 72 h without detectable liver injury. The same group reported that NEVLP decreased liver injury of DCD liver grafts when compared with cold static storage alone (92, 169, 194-196). Of note, the authors observed a worrisome increase of liver injury and decrease of graft function when normothermic whole blood perfusion was combined even with a short cold ischemia time (195). Schoen et al. (197) compared 1 h warm ischemia followed by 4 h cold static preservation with livers exposed to 1 h warm ischemia plus 4 h normothermic ex vivo perfusion. Liver transplantation was performed in both groups. Schoen et al. (197) found that normothermic perfusion without cold storage reduced liver injury, improved liver function and increased survival after transplantation. None of the above studies investigated bile duct injury as the key problem of DCD liver transplantation.

Cold liver perfusion has also been explored as preservation technique. Guarrera et al. (121, 198, 199) performed cold perfusion of the HA and PV without oxygenation. The authors used the same low pressure of 4–6 mmHg for both the HA and PV. No benefits of hypoxic cold perfusion were observed in pig livers. However, in a case control study with 20 human liver transplants using low risk donors the same authors observed a decrease of serum transaminases after transplantation when cold perfusion was compared to cold static preservation. de Rougemont et al. (128) examined the effects of a short (1

h) oxygenated cold perfusion prior to pig liver transplantation. Livers were exposed to 1 h warm ischemia followed by 7 h cold static preservation or 1 h warm ischemia plus 6 h cold preservation and 1 h oxygenated cold perfusion. After liver transplantation, AST levels were similar in both groups. Median pig survival after transplantation was marginally increased by oxygenated perfusion from 5 to 8 h. Bile duct injury was not investigated in either of these studies.

In our model of normothermic ex vivo acellular liver perfusion we did not use blood cells or serum in our perfusate to minimize the potential adverse effects of mediators of inflammation during perfusion. In addition, using a blood free perfusate potentially minimizes possible risks of intrahepatic clotting, breakdown of erythrocytes and infection risks associated with a blood perfusate. Our study was designed to simulate a clinical scenario when organs are retrieved at a remote donor hospital and brought on ice to the transplant center to initiate normothermic perfusion. Therefore, we added 4 h cold static storage to the normothermic perfusion group as it would be required for a clinical trial. The combination of warm ischemia during DCD retrieval followed by cold static storage represents specific challenges for normothermic ex vivo perfusion and blood products in the perfusion solution might induce reperfusion injury during the normothermic ex vivo perfusion. Our perfusate was free of any blood or serum products and we did not detect any injury during the normothermic oxygenated perfused preservation despite previous substantial ischemic injury of the grafts.

This study also examined specifically bile duct injury which is the current key limitation of DCD liver transplantation. No bile duct injury occurred after ischemia alone without reperfusion. As predicted by the clinical experience, we observed severe bile duct injury in livers exposed to 1 h warm ischemia and 4 or 12 h cold static storage plus blood reperfusion. Hashimoto et al. (200) hypothesized that bile duct injury after DCD liver transplantation is induced by reduction of arterial flow in DCD liver grafts. In support of this prediction, we found that HA perfusion of the liver parenchyma was decreased by 50% in DCD liver grafts after 12 h blood reperfusion. Preservation of DCD grafts by first cold storage and then normothermic perfused preservation improved HA flow, decreased bile duct hypoxia and prevented bile duct injury. These findings indicate that bile duct injury occurs only after reperfusion and that modification of the preservation conditions can rescue bile ducts despite severe ischemia during DCD retrieval.

The mechanisms contributing to improved arterial flow and decreased bile duct injury after normothermic perfused preservation were not examined in this study, as this was an

unexpected finding. It is possible that the warm perfusion with the vasodilator epoprostenol resulted in an improved arterial flow and either avoided clot formation or removed arterial microthrombus. Other potential mechanisms include the promotion of ATP generation during normothermic perfused preservation or a direct protective effect on either vascular endothelial cells or bile duct mucosa.

Acellular normothermic perfusion has also been reported to improve reperfusion injury in lung transplantation. Cypel et al. (201-205) demonstrated in animal models as well as in a human clinical trial that normothermic lung perfusion is superior to cold static storage and can be used to assess lung function prior to transplantation. The authors also implemented strategies to improve marginal lungs during normothermic perfusion prior to transplantation.

Our study has several limitations. We used a model of blood reperfusion to simulate transplantation. It is possible that blood reperfusion results in different degrees of liver injury than in vivo reperfusion during transplantation. The observed benefits in hepatocyte and bile duct protection may not directly translate into a transplant model. Thus, NEVLP for DCD liver grafts must first be confirmed in a transplant survival model. Additional studies should focus on mechanisms of bile duct protection by normothermic perfused preservation. In addition, ALT as a marker of hepatocyte injury might be less reliable in the ex vivo reperfusion model. It is possible that NEVLP washed out transaminases during normothermic preservation and therefore contributed to the low ALT levels during ex vivo blood reperfusion.

In conclusion, this study indicates that acellular normothermic perfusion improves markers of bile duct injury and enhances arterial perfusion of DCD grafts in a model of blood reperfusion. Acellular normothermic perfusion is a promising new technique for the storage, assessment and repair of DCD liver grafts.

5.2 Subnormothermic Acellular Ex Vivo Liver Perfusion in Pig Livers Retrieved After Cardiac Death

This study demonstrates that SNEVLP protects DCD liver grafts against sinusoidal EC injury and decreases bile duct necrosis after liver transplantation. DCD liver grafts represent a large donor pool, which could significantly improve the current donor shortage. Unfortunately, DCD liver transplantation is associated with a high risk (20%-40% of cases) for ischemic-type bile duct injury. (206-210) This has resulted in strict selection criteria for DCD grafts, and as a result, they are often declined based on donor age or

warm and cold ischemia times. A better preservation technique is required to protect bile ducts in DCD liver grafts and to make this donor group more broadly available for liver transplantation. (135, 206-212)

Normothermic ex vivo liver perfusion or SNEVLP has been previously used to protect DCD liver grafts against preservation injury. Brockmann et al. (169) compared normothermic ex vivo liver perfusion with CS in DCD liver grafts. Using diluted blood as a perfusate, they reported a significant reduction of liver injury and improved survival with warm perfused storage. In their model, the authors used only 1 hour of CS before warm perfusion and did not investigate bile duct injury. The same group determined that the protective effect was lost when the length of CS before warm-perfusion preservation was prolonged. (195, 213) Similarly, Schön et al. (197) demonstrated that normothermic ex vivo liver perfusion versus CS improved survival after liver transplantation with DCD grafts. Similarly to Brockmann et al., (169) this group did not use CS before their normothermic perfusion model. Fondevila et al. (119) used combined ECMO in vivo plus normothermic ex vivo perfusion in pig DCD liver grafts. The authors demonstrated that normothermic perfusion improves the outcome of DCD liver transplantation if CS can be avoided. Using ECMO during DCD organ retrieval is an interesting approach and could supplement normothermic ex vivo perfusion by minimizing organ cooling before normothermic perfusion of DCD grafts.

Cold nonoxygenated or oxygenated perfusion systems have been used by others. (118, 128, 158, 214) Cold perfusion at 4°C offers the advantage for the liver of minimal oxygen requirement and, therefore, prevents reperfusion injury during the ex vivo perfusion. Also, failure of the perfusion system during transport would not automatically result in a loss of the liver graft. Unfortunately, the effects of cold perfusion on bile duct injury have not been investigated so far in a large animal model. Schlegel et al. (158) demonstrated a protective effect on biliary tissue in a rodent transplant model without arterial reconstruction. However, the role of hepatic arterialization in biliary injury is considered to be of superior importance in the occurrence of ITBS. (200) In addition, the low metabolism at cold temperatures precludes an assessment of graft function and makes the application of protective interventions more difficult. De Rougemont et al. (128) used 1 hour of oxygenated cold perfusion before DCD liver transplantation. Although no difference in transaminase release was observed after transplantation, the mean animal survival was slightly increased from 5 hours to 9 hours after transplantation.

Currently, transporting grafts from the donor hospital to the transplant center requires static cooling of the liver grafts for several hours. Portable perfusion devices that allow avoidance of any cooling period are attractive solutions for preventing cold ischemic injury. Such devices are in development but have not yet reached routine clinical practice. Meanwhile, transportation of the graft to the transplant center with CS followed by warm perfusion would simplify the logistics of the process. Hence, our study was designed to mimic a clinical scenario in which the grafts were to be transported from the donor hospital to the transplant center in CS before SNEVLP. Therefore, we included 4 hours of CS before the start of SNEVLP in our protocol. Because warm perfusion is technically challenging and a failure of perfusion after skin incision in the recipient would seriously jeopardize the patient's life, we added an additional 3 hours of CS after SNEVLP for the time of the recipient's preparation and hepatectomy.

Previously published studies have used blood or diluted blood as a perfusate for warm ex vivo perfusion. Blood-derived perfusate solutions have several disadvantages. Blood contains mediators of reperfusion injury, such as leukocytes, platelets, and cytokines. Thus, warm ex vivo perfusion after a period of CS might induce reperfusion injury, which could explain the inferior outcome for combined CS and warm ex vivo perfusion as mentioned previously.

In previous experiments, we used a completely acellular normothermic perfusion solution without any blood components for normothermically perfused organ preservation. (215) Using whole-blood ex vivo reperfusion as a model for transplantation, we found decreased bile duct injury in DCD grafts compared with CS livers. However, acellular perfusion was associated with decreased liver function and inferior long-term survival after pig liver transplantation. Therefore, in our current study, we changed our perfusion technique by using leukocyte-depleted, washed erythrocytes as oxygen carriers. In contrast to other solutions, our ex vivo perfusion solution was designed to minimize contamination with leukocytes, platelets, and serum during the ex vivo perfusion. Our albumin based perfusate (Steen solution) with its osmotic properties replaced the cytokine-rich plasma fraction. Along with the addition of other active substances, the solution was able to decrease the activation of the inflammatory cascade substantially. Adding washed, leukocyte-depleted, and serum-free erythrocytes as oxygen carriers to the perfusate improved liver function after transplantation with similar protective effects on bile ducts in comparison with the acellular perfusate. A second important finding in this study is the protection of ECs by SNEVLP preservation. It is possible that decreased EC injury improves

arterial perfusion of the biliary tree, which could result in protection of bile ducts against ischemic injury.

Hepatocyte injury was similar in the CS and SNEVLP groups. Although AST levels were decreased early after transplantation in pigs with SNEVLP versus CS, peak AST levels were similar at day 1, with a similar pattern of decline afterward. This indicates that hepatocyte injury was delayed, but not decreased, by SNEVLP preservation. However, primary nonfunction is rare in clinical DCD liver transplantation, and the high incidence of ITBS is the major obstacle for the extensive use of DCD liver grafts. Our finding that bile duct injury is reduced in SNEVLP versus CS DCD grafts could, therefore, represent an important advantage of warm-perfusion preservation of DCD grafts in comparison with CS.

This study has several limitations. First, mechanisms of protection were not investigated in detail. Further studies are needed to investigate the impact of warm-perfusion preservation on the bile duct blood supply and injury after transplantation. Furthermore, the protective effect was limited to bile duct protection without reducing hepatocyte death and without improving graft function. The experimental design with 45 minutes of WI and 10 hours of preservation, accounting for only limited hepatocyte injury, allowed graft recovery and pig survival in both the SNEVLP and CS groups. Longer ischemia times might be required to investigate whether SNEVLP has an effect on hepatocyte injury and function as well as graft survival. We chose 4 hours of CS before SNEVLP and 3 hours of CS after SNEVLP in our model. In clinical practice, these CS times could often be reduced, and prolonged CS before warm perfusion might not be required in a large proportion of cases. Finally, we cannot exclude additional mechanisms present in human grafts in comparison with the porcine model.

In summary, this study demonstrates that organ preservation with combined CS and subsequent SNEVLP protects DCD liver grafts against ischemic-type bile duct injury and reduces EC death. Reducing the incidence of ITBS could allow us to use DCD liver grafts better and to increase the donor pool for liver transplantation.

5.3 Literature search

The introduction of Ex-Vivo Liver Machine Perfusion into clinical practice has led to further progress in the field of liver transplantation. It is attempting to increase the donor pool and consecutively to reduce death on waiting list. The last years show an

increasing interest and research - both clinical and experimental - in the field of machine perfusion, leading to several studies in this area.

One of the greatest benefits of MP over the clinical golden standard of CS is the ability to evaluate the viability and functionality of the graft during preservation. Several markers have been proposed by different studies, such as AST, ALT and LDH levels in the perfusate (most commonly used markers of hepatocellular damage), pressure/flow characteristics, lactate conversion and bile production. Additionally, oxygen consumption, factor V (indicators of metabolic and synthetic function), and hyaluronic acid -which is a marker of endothelial injury- have also been used as endpoints for graft assessment. Although the aforementioned markers have been effective indicators of graft quality, many other biomarkers have also been proposed, including glucose metabolism, pH and microRNA levels (94, 216, 217). There is, currently, no consensus on which of the biomarkers can most accurately predict the transplantation outcome and indicate histological and functional damage of the liver (7, 15). Consequently, further investigation is required to establish reliable measures of high diagnostic potential to accurately assess graft viability and functionality during MP and determine the optimum transplantation outcome.

Since each MP technique refers to a different working temperature, it is characterized by different metabolic requirements that need to be fulfilled at the expense of added complexity in the system. This trade-off between functionality and complexity greatly affects the selection of the machine perfusion technique, as well as the combination of the machine perfusion parameters. The low temperature of HMP minimizes the metabolic requirements of the liver and allows the development relatively simple devices. This is confirmed by the results of the literature study, which showed that the majority of HMP systems employed an acellular oxygenated perfusate, with single-circuit perfusion and continuous flow. On the contrary, a significantly more complex configuration is necessary in NMP devices. The increased metabolic requirements of the liver need to be met, and for this reason a cellular perfusate is needed alongside with a dual-circuit perfusion. In addition, the type of flow is usually pulsatile, and in many cases it differentiates between PV and HA. These characteristics of NMP can better emulate the physiological environment of the liver compared to HMP, and for this reason longer preservation periods have been achieved with this technique. However, the greatest disadvantage of NMP systems is their complexity, which plays a crucial role in the implementation of the technique in the real clinical world. SNMP systems appear to be more versatile and offer a

compromise between functionality and simplicity. Configurations at subnormothermic temperature have shown promising results towards long-term perfusion in an attempt to reduce the complexity of NMP and improve the functionality and effective duration of HMP systems. Consequently, the practical and logistical value of SNMP systems appear to be greater than this of NMP devices, while longer preservation periods are accomplished, compared to the limited preservation times that are observed in HMP.

Regarding HMP, the temperature range is defined between 1-22ºC (15), as is also evidenced by the studies included in the present review (**Table 4**). In principle, at lower temperatures the metabolic activity is reduced to a greater extent, whereas the viscosity of the perfusate increases. As a result, lower HMP temperatures are associated with higher vascular resistance and increased risk of endothelial damage (91), as mentioned. As the damage becomes more pronounced when prolonged perfusion is applied, ex-situ HMP experiments are limited, in terms of duration, from 2 to 24 hours (15, 77). This comes in accordance with **Table 4**, where the short duration of HMP experiments is clearly demonstrated. UW-gluconate is the major perfusate utilised in HMP studies, despite the fact that the presence of HES is a matter of controversy and there have been suggestions to either replace it with PEG or completely omit it to reduce the perfusate viscosity (77, 131). Dual perfusion is typically selected to accomplish a better representation of the physiological environment ex-vivo. During HMP, oxygen consumption of hepatocytes is decreased, while oxygen saturation of the hypothermic perfusate is greatly higher than in normothermic temperatures (131, 218). Therefore, single HMP effectively achieves adequate oxygenation of the graft. Typically, HMP systems that do not use oxygenation require dual-vessel perfusion, as shown in **Table 4**. The low metabolic needs of the graft allow either oxygenation or HA perfusion to be omitted, however not both. Oxygenated perfusion has yielded better results in terms of graft survival in the recipient. The viability assessment in HMP is accomplished with the abovementioned biomarkers and endpoints, however, it should be noted that due to the reduced metabolic activity, real-time viability evaluation is difficult and further research on the topic is required (15). Last but not least, one of the major advantages of HMP is the ease of application after static cold storage. Many studies apply end-ischemic HMP after an initial SCS and -despite the fact that it is not used for extended preservation- it can also be used for a short period of time for graft resuscitation immediately before transplantation (26).

NMP systems are typically more technically complicated as they aim in recreating preservation conditions as close to the physiological environment as possible. At normothermic temperature, the fully functional metabolism of the graft requires to be supported by nutrient and sufficient oxygen delivery. Generally, full blood or RBC-based perfusates are utilized (**Table 5**), with RBCs as oxygen carriers. Despite blood-based NMP supports the graft functionally, it exhibits certain drawbacks, such as RBC hemolysis, immune-mediated phenomena as well as logistical complexities regarding the use of cross-matched blood (137). As opposed to HMP devices, all NMP systems are dual-circulating setups and perfuse both PV and HA, in order to better emulate the physiological conditions (80). The results in **Table 5** are consistent with this NMP characteristic, while they indicate that the lack of consensus regarding the benefits of pulsatile over continuous flow has led research groups to use both types of perfusion. The full metabolic support provided by NMP leads to preservation of alive and functional grafts for relatively long periods of times, as is highlighted in the outcome of the studies included in **Table 5**. In addition, the full metabolic activity facilitates the real-time functional assessment of the liver with the use of selected biomarkers. Of note, Friend et al. achieved successful NMP of porcine livers for 72 hours (95); nonetheless, such an extended period of NMP has not been reported by any other experimental study. Finally, despite the safety and feasibility of NMP as well as its superiority over CS is demonstrated in the literature, there are still certain difficulties that hinder its widespread clinical application. NMP systems are technically complex and require adequately trained personnel for their operation; in contrast to cold preservation techniques, any technical failure NMP leads to warm ischemia time, which can be detrimental for the graft (15). Moreover, perfusion in warm environments is more susceptible to bacterial contamination and in some cases administration of antibiotics may be necessary (15). Transportability is of great importance, as it can minimize CIT which damages the graft (119, 195), however it still remains a technical obstacle in most cases (80).

SNMP has emerged as an alternative compromise between NMP and HMP. The intermediate temperature levels allow lower metabolic activity, while the metabolic function of the graft is maintained at sufficient levels (96, 97). Thus, SNMP allows real-time viability assessment of the graft with the use of the investigated biomarkers in the perfusate and bile, offering a balance between the harmful effects of hypothermia and the system complexity that comes along with full metabolic demands at normothermic temperatures (28). As shown in **Table 6**, SNMP is performed with an oxygenated perfusate

with encouraging results in terms of liver injury and graft synthetic function at 20ºC. At this temperature, the metabolic oxygen demands of the liver are met by active oxygenation and oxygen carriers can be omitted (219). This can reduce the need for strict and accurate temperature control as well as eliminate the necessity of oxygen carriers in the perfusate, which leads to essentially simplified and less costly MP systems (15). Indeed, with the exception of Fontes et al. (191), who reported effective liver SNMP using Hemopure as an acellular oxygen carrier, the included studies have accomplished successful SNMP by obviating oxygen carriers. Acellular perfusates are utilised, with Krebs-Henseleit (KH) and UW-G solutions being the most characteristic examples. Fontes et al. (191) also performed dual-circuit, pulsatile HA/continuous PV SNMP as opposed to the most commonly used single-vessel continuous SNMP showing promising results. Overall, it can be deducted that SNMP is a viable and effective alternative of NMP and HMP, capable of supporting livers with minimal injury and maintain their functionality, while simplifying the MP configuration and lowering its cost.

Overall, the present literature review investigates both clinical and technical aspects of MP and shows that MP is an effective method for successful ex-vivo liver preservation and is performed by means of complex and technically demanding devices. Although there have been several commercially available MP devices for specific techniques and conditions, the vast majority of the work done in the field is experimental and ongoing research on how to optimize this preservation method is performed by many groups worldwide. In this direction, it is clear that further research needs to be conducted towards establishing objective and universal endpoints for viability evaluation of the graft. In addition, widespread clinical application of MP will be facilitated by the development and introduction of protocols and regulations regarding the application of MP. Such implementations, alongside with the safety, feasibility and outcome benefit of MP over SCS, can pave the way for wide introduction of dynamic preservation methods into clinical practice. From a technical standpoint, the simplicity of the device plays an important role in its function and operation. The elements that comprise a MP configuration add both functionality and complexity in the system, and a compromise is necessary to be achieved, depending on the purpose of the preservation. Alongside, the assembly and operational costs are decreased, while complexity decreases.

There have been several experimental machine perfusion setups through the history of the field, which combined different perfusion settings for each technique. Many groups have developed devices with different operational parameters to examine their

effect on the preservation outcome. The unique characteristics and requirements of each technique have been discussed; however, nowadays it has been established that a perfusion environment as close as possible to the physiological is beneficial for graft preservation. Thus, dual, oxygenated and pulsatile perfusion is the combination that is mainly adopted in the most recent studies, as it emulates the physiological conditions of the liver and its superiority over other perfusion types has been established.

Finally, it is evident that long term perfusion is more challenging and difficult to achieve. Taking into consideration the favorable results of SNMP in terms of graft quality as well as the simplicity compared to NMP, subnormothermic perfusion appears to be a promising solution for the achievement of prolonged liver preservation. This has been confirmed by the recent promising results of Clavien et al., who reported successful preservation of injured human livers for 1 week (220). However, the ideal subnormothermic temperature for the achievement of effective long-term liver machine perfusion has not been determined yet. Thus, further investigation is needed for the selection of a temperature, in which the metabolic activity of the liver is reduced but not completely ceased. Thus, liver enzymes can be kept active so that ATP synthesis is not stopped, while the metabolism and oxygen demand is decreased to a great extent compared to NMP (122).

5.4 Outlook

There is strong commercial activity in the field of liver perfusion, indicating an unmet need and the anticipation of a growing commercial market that will be using the insights gained from, among many others, the experiments described in this thesis:

LifePort Liver Transporter (166) by Organ Recovery Systems
Itasca, IL USA:
SPS-1® (UW Solution):
Static Preservation Solution
to be cooled prior to use

Constituents	Amount / 1000 mL
Hydroxyethyl Starch (HES)	50 g
Lactobionic Acid (as Lactone)	35.83 g
Potassium Phosphate monobasic	3.4 g
Magnesium Sulfate heptahydrate	1.23 g
Raffinose Pentahydrate	17.83 g
Adenosine	1.34 g
Allopurinol	0.136 g
Glutathione (reduced form)	0.922 g
Potassium Hydroxide	5.61 g

LifePort Liver Transporter® is in the process of securing US and European regulatory registrations, and is not yet approved for clinical use.

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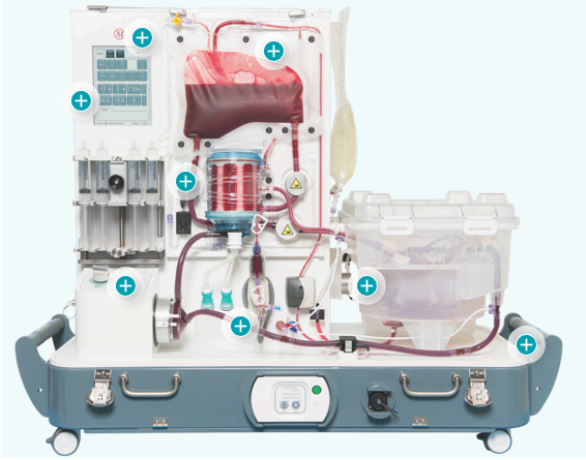
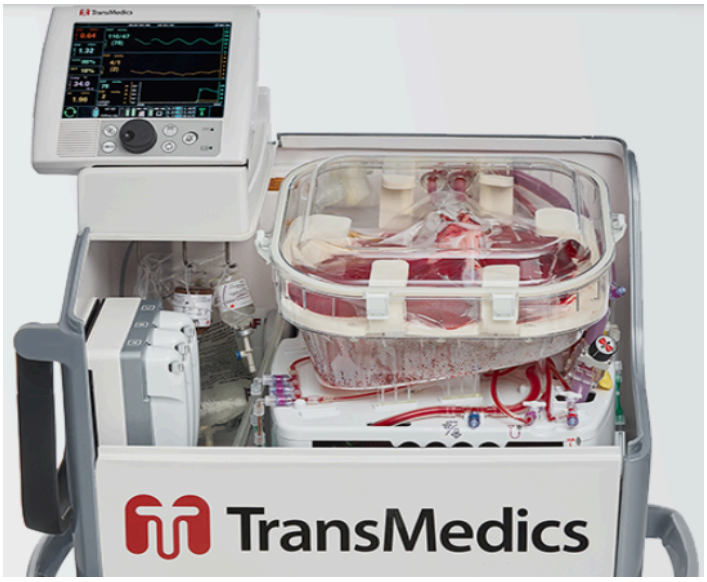
OrganOx metra® by Organox, Oxford, UK (168):



"During normothermic machine perfusion with the OrganOx metra®, the donor liver is continuously perfused with oxygenated blood, medications and nutrients at normal body temperature and near physiological pressures and flows. This means the liver is functional throughout preservation, enabling functional assessment and evidence-based decisions on whether to transplant a donor organ."

OCS™ Liver by Transmedics, US (167):



" The OCS Liver is an FDA approved device for DCD and DBD donor Livers."

DCD livers < 55 years old with < 30 mins warm ischemia time and macrosteatosis < 15%.

Successful Completion of 300 Patient US FDA Pivotal Trial (ClinicalTrials.gov Identifier: NCT02522871) for Expanded Criteria and DCD Donor Livers

Liver Assist by XVIVO B.V., Groningen, Netherlands (165)

"The most important feature of all our devices is that they allow for true oxygenated perfusion. Multiple (clinical) research projects have already proven that the addition of oxygen to the perfusion fluid has positive effects on the organ(s) as well as the transplant results. Livers for transplant for example show that just hypothermic oxygenated perfusion is improving graft quality and survival and prevents for ischemic cholangiopathy after transplant.

Liver4Life by WYSS, Zurich, Switzerland (221) offers an even more daring perspective for the use of normothermic ex-vivo liver perfusion:
"The Wyss Zurich project will develop a novel therapeutic strategy for liver regeneration consisting of: i) surgical resection of a small healthy piece of the liver from the patient; ii) growth of this piece outside of the body in a perfusion machine until a sufficient size is reached; iii) retransplantation of the regenerated liver to the original patient while removing the remaining diseased part. Current perfusion systems are not able to keep a liver alive outside of the body for a sufficient time to allow growth and regeneration to occur. The challenging aim of the project is to extend the viability of liver tissue outside of the body up to five days and allow its growth.

As becomes clear from the data and images provided by these companies, there are still different perfusion solutions in use, which in part include blood with its accompanying risks of inflammatory mediators, eg cytokines, chemokines or leukotrienes, contained and secreted by blood components such as leukocytes and platelets. The oxygen-carrying capacity of hemoglobin, which is superior to artificial carriers used in acellular solutions, appears to outweigh the disadvantages associated with its use.
How to address this conflict in the future, with the aim of providing perfect perfusion without the risk that are presently posed by both blood or acellular solutions? Increased knowledge of the detailed flow of both blood and bile inside the hepatic lobules will inform better perfusion methodology to provide a more equal penetration of the perfusion liquid through the highly complex system of canaliculi that comprises the liver. Increased knowledge of molecular processes that lead to the death of hepatocytes, non-parenchymal liver cells such as hepatic stellate cells and cholangiocytes, and liver stem cells by apoptosis, necrosis or pyroptosis during IRI will also enable clinicians to add compounds that will specifically reduce these death cascades during the liver preservation stage.
The knowledge and experience gained through the experiments and the literature review in this thesis will enable further research aimed at improving perfusion solutions and flow dynamics for liver perfusion, which is essential to expend the donor pool for human liver transplantation. Considering future challenges posed by increased levels of metabolic and substance-induced end-stage liver disease, the future of ex vivo perfusion for liver transplantation is bright, and it is acellular.

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6. Conclusion

As becomes clear from the data and images provided by these companies, there are still different perfusion solutions in use, which in part include blood with its accompanying risks of inflammatory mediators, eg cytokines, chemokines or leukotrienes, contained and secreted by blood components such as leukocytes and platelets. The oxygen-carrying capacity of hemoglobin, which is superior to artificial carriers used in acellular solutions, appears to outweigh the disadvantages associated with its use.

How to address this conflict in the future, with the aim of providing perfect perfusion without the risk that are presently posed by both blood and acellular solutions? Increased knowledge of the detailed flow of both blood and bile inside the hepatic lobules will lead to better perfusion methodology to provide a more equal penetration of the perfusion liquid through the highly complex system of canaliculi that comprises the liver. Increased knowledge of molecular processes that lead to the death of hepatocytes, non-parenchymal liver cells such as hepatic stellate cells and cholangiocytes, and liver stem cells by apoptosis, necrosis or pyroptosis during IRI will also enable clinicians to add compounds that will specifically reduce these death cascades during the liver preservation stage.

The knowledge and experience gained through the experiments and the literature review in this thesis will enable further research aimed at improving perfusion solutions and flow dynamics for liver perfusion, which is essential to expend the donor pool for human liver transplantation. Considering future challenges posed by increased levels of metabolic and substance-induced end-stage liver disease, the future of ex vivo perfusion for liver transplantation is bright, and hopefully will be acellular.

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7. Summary

Increasing indications for liver transplantation led to a shortage of liver grafts across the world, resulting in increasing waiting list mortality. Ex Vivo Liver Perfusion provides a novel preservation, which might replace the current preservation by cold storage. The aim of utilizing this technology is to increase the liver donor pool and consecutively reduce waiting list mortality. Beyond the approach of better preservation, the method offers the possibility of organ assessment and potential organ repair prior to transplantation.

The experimental part of the thesis aimed to establish a large animal model to investigate the impact of temperature and perfusion solution during *ex-vivo* liver perfusion on the quality of the preserved organ. The impact of acellular perfusion solution, i.e. a cell free solution not containing blood as oxygen carrier, was of particular interest to avoid additional immunological effects. Experimental conditions were chosen to best mimic the conditions of liver transplantation. Several parameters of hepatic injury were investigated as potential biomarkers to assess the impact of ischemia-reperfusion-injury on the quality of the liver graft and thus future function and post-transplantation survival with a special focus on ischemic type biliary injury.

The first experimental setup showed that Normothermic *Ex Vivo* Liver Perfusion (NEVLP) with an acellular perfusion solution resulted in improved arterial perfusion, decreased hepatocellular damage, and reduced markers of bile duct injury in a pig model that mimics donation after cardiac death (DCD) liver transplantation. Markers of liver function as bilirubin, phospholipids and bile salts were fivefold decreased in cold stored (CS) versus NEVLP grafts, while LDH, a marker of hepatocellular damage, was six fold higher. Following transplantation, mean serum AST level, as a marker of damage was higher in CS versus NEVLP. Furthermore, NEVLP improved hepatic artery perfusion and decreased markers of bile duct injury in DCD grafts.

The second study compared cold storage (CS) with combined CS and Sub-Normothermic *Ex Vivo* Liver Perfusion (SNEVLP) for the preservation of donation after cardiac death (DCD) liver grafts in a model of pig liver transplantation. The effects of SNEVLP in DCD grafts on hepatocyte, sinusoidal endothelial cell (EC), and bile duct injury after transplantation were assessed. Post transplant SNEVLP animals showed lower serum AST and serum bilirubin levels in comparison to cold CS animals. In addition, LDH in bile fluid was lower in SNEVLP pigs compared to CS pigs. Bile duct histology revealed

severe bile duct necrosis in 3 of 5 animals in the CS group but none in the SNEVLP group.

The literature review showed that even in 2023, the ideal temperature and timing for machine perfusion remain controversial. Although several successful clinical trials using normothermic machine perfusion (NMP) have been reported, NMP has the problem that physiological preservation temperatures necessitate higher oxygen supply. Dissolved oxygen alone is insufficient, and in clinical studies, human blood or blood fractions are still used.

It should be stressed though that normo- and hypothermic Machine Perfusion is used for different purposes. While the hypothermic is used as short-term conservation, the normothermic setting is essential if longer preservation is required, e.g. for assessment and potential repair of organs, as the activity of enzymes is essential.

The sub-normothermic setting is based on the idea to maintain liver function and consecutively allow organ assessment, while the reduced temperature reduces oxygen consumption. The latter should ideally allow using a cell free perfusion solution.

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9. Bibliography of the candidate´s publications

I. List of original publications within the topic of the PhD thesis:

Knaak Jan M., Spetzler Vinzent N., Goldaracena Nicolas, **Boehnert Markus U**., Bazerbachi Fateh, Louis Kristine S., Adeyi Oyedele A., Minkovich Leonid, Yip Paul M., Keshavjee Shaf, Levy Gary A., Grant David R., Selzner Nazia, Selzner Markus Subnormothermic Ex Vivo Liver Perfusion Reduces Endothelial Cell and Bile Duct In- jury After Donation After Cardiac Death Pig Liver Transplantation
LIVER TRANSPLANTATION 20: 11 pp. 1296-1305., 10 p. (2014)
Journal Article (Article) | Scientific |
Journal subject: Scopus - Surgery Rank: D1
Journal subject: Scopus - Hepatology Rank: Q1
Journal subject: Scopus - Transplantation Rank: Q1
IF: 4,241

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Normothermic Acellular Ex Vivo Liver Perfusion (NEVLP) Reduces Liver and Bile Duct in DCD Liver Grafts
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Boehnert M. U., Yeung J. C., Bazerbachi F., Knaak J. M., Selzner N., McGilvray I. D., Rotstein O. D., Adeyi O. A., Kandel S. M., Rogalla P., Yip P. M., Levy G. A., Keshavjee S., Grant D. R., Selzner M.
Normothermic Acellular Ex Vivo Liver Perfusion Reduces Liver and Bile Duct Injury of Pig Livers Retrieved After Cardiac Death
AMERICAN JOURNAL OF TRANSPLANTATION 13: 6 pp. 1441-1449., 9 p. (2013)
Journal Article (Article) | Scientific
Journal subject: Scopus - Pharmacology (medical) Rank: D1
Journal subject: Scopus - Transplantation Rank: D1
Journal subject: Scopus - Immunology and Allergy Rank: Q1
IF: 6,190

II. List of original publications not relating to the topic of the PhD thesis:

Takagi Kosei; Domagala Piotr; Polak Wojciech G; Ijzermans JNM; **Boehnert Markus U.**
Right posterior segment graft for living donor liver transplantation: A systematic review
TRANSPLANTATION REVIEWS 34: 1 Paper: 100510, 7 p. (2020)
Journal Article (Survey paper) Scientific
Journal subject: Scopus - Transplantation Rank: Q1 **IF: 3,943**

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APPENDIX

Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/Flow-controlled	Major End-points	Outcome
Dutkowski et al. (1998) (88)	Rat	HBD	Continuous	1. Pulsatile HMP (N=6) 2. Continuous HMP (N=6) 3. CS (N=6)	3-6	10	Modified UW solution (Yes)	PV	1.Pulsatile 2.Continuous	Flow	ATP, AMP, lactate, LDH, glutathione, energy charge	ATP levels were higher in HMP groups (Highest in pulsatile) Higher AMP in CS. Lower lactate and LDH in HMP. Pulsatile HMP increased energy charge.
v.d. Plaats et al. (2006) (118)	Pig	HBD	Continuous	1. HMP (N=6) 2. CS (N=2)	0-4	24	UW MP solution (Yes)	Dual	HA: Pulsatile PV: Continuous	Pressure	Oxygen, LDH, histology	pO_2 was constant in HA; in PV it increased until 8h and then remained constant. LDH increased. No major edema in the microvascular system.
Guarrera et al. (2010) (121)	Human	DCD (44.3±6.5 min)	End-ischemic (CIT= 9.4±2.1 h)	1. HMP (N=20) 2. CS (N=20)	0-4	3-7	Vasosol (No)	Dual	Continuous	Flow	AST, ALT, LDH, EAD, total bilirubin, creatinine	HMP group had less EAD (5% vs 25%) and shorter hospital stay. AST, ALT, TBil, SCr were lower in the HMP group. Less biliary complications

												in the HMP group.
Olschewski et al. (2010) (147)	Rat	DCD (60 min)	Continuous	1. HMP4 (N=5) 2. HMP12 (N=5) 3. SNMP (N=5) 4. CS (N=5)	4 12 21	6	Lifor solution (Yes)	PV	Continuous	Flow	ALT, bile flow, histology, PV resistance	SNMP group showed increased bile flow, post-ischemic bile recovery and lower PV resistance than all other groups. HMP groups had less ALT release than SNMP and CS. Low degree histological damage in all groups.
Schlegel et al. (2013) (158)	Rat	DCD (60 min)	End-ischemic (CIT=4h)	1. HOPE (N=10) 2. CS (WI) (N= 10) 3. CS (no injury) (N=10)	4	1 4 0.75	Modified starch-free UW solution (Yes)	PV	Continuous	Pressure	AST, ALT, Factor V, tissue ATP, histology	HOPE led to less ATP and ALT release, higher factor V and ATP levels. Histological examination showed that HOPE led to less apoptosis and protected from biliary fibrosis.
Dirkes et al. (2013) (186)	Pig	HBD	Continuous	HMP (N=3)	10 ± 0.5 (average)	20	Belzer MP solution (Yes)	PV	Pulsatile	Pressure	LDH, AST, bilirubin, histology, pO_2, pCO_2	AST and LDH increased, bilirubin remained low. No portal edema or parenchymal necrosis. pCO2 decreased, pO2 increased

Dutkowski et al. (2014) (222)	Human	DCD (26-43 min)	End-ischemic (CIT=1.4-3.5h)	1. HOPE (N=8) 2. DBD, matched (N=8)	10	1.4 – 3.5 (median =2)	UW gluconate solution (KPS) (Yes)	PV	Continuous	Pressure	AST, ALT, ICU stay, ALP, bilirubin, gamma-GT	Low release of AST, ALT in HMP livers and short ICU stay. ALP, bilirubin and gamma-GT remained low in perfused DCD and DBD grafts.

Table 4. *Results Table for Hypothermic Machine Perfusion.*

Table 4. *Continued*

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Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/Flow-controlled	Major End-points	Outcome
Guarrera et al. (2015) (142)	Human	ECD	End-ischemic (CIT= 9.3±1.6h)	1.HMP (N=31) 2.CS (N=30)	4-8	3-7	Vasosol (No)	Dual	Continuous	Flow	PNF, EAD, AST, ALT, TBil, SCr	AST, ALT, TBil and SCr were lower in HMP group. 1 PNF and 6 EAD incidents in HMP group compared to 2 and 9 in CS group respectively. Less biliary complications in HMP.
Dutkowski et al. (2015) (223)	Human	DCD (31-40 min)	End-ischemic (CIT=2.4-4.4h)	1. HOPE (N=25) 2. CS, DCD (N=50) 3. CS, DBD (N=50)	10	1-2	UW gluconate (KPS-1) (Yes)	PV	Continuous	Flow	ALT, AST, PNF, bilirubin	Lower ALT, AST, bilirubin in HOPE group. No PNF in HOPE group, 6% in CS, DCD. HOPE group had less intrahepatic cholangiopathy than CS and all endpoints were comparable to DBD.
Jia et al. (2015) (224)	Rat	HBD	Continuous	1. HMP (N=18) (1a. Saline, 1b.UW, 1c. HTK) 2. CS (N=18) (2a. Saline, 2b. UW, 2c. HTK)	4 0-4	6	1. Saline solution (No) 2. UW solution (No) 3. HTK solution (No)	PV	Continuous	Flow	ALT, AST, LDH, histology, MDA, ATP	HMP decreased ALT, AST, LDH and had lower MDA than CS regardless of perfusate. UW reduces edema most efficiently, HTK maintains ATP levels best. UW showed higher vein resistance and ATP consumption than HTK.
v. Rijn et al. (2017) (126)	Human	DCD (23-43 min)	End-ischemic (CIT=308-376min)	1. DHOPE(N=10) 2. CS (N=20)	10	2.1-2.3	UW-MPS (Yes)	Dual	HA: Pulsatile PV: Continuous	Pressure	ALT, ALP, GGT, TBil, graft survival	Higher 6-month, 1-year graft survival and patient survival in DHOPE (100% vs 80, 57, 85%) ALT, ALP, GGT, TBil lower in DHOPE, while ATP increased 11-fold.
Kanazawa et al. (2020) (225)	Pig	DCD (60 min)	Continuous	1. HMP (N=5) 2. SNMP (N=5) 3. CS (N=5)	8 22	4	Modified UW gluconate solution (Yes)	Dual	Continuous	Pressure	AST, LDH, ATP, histology	AST, LDH, lactate and sinusoidal epithelium injury was significantly higher in CS group. ATP levels were higher in SNMP and HMP.

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Table 5. *Results Table for Normothermic Machine Perfusion*

Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/Flow-controlled	Major End-points	Outcome
Imber et al. (2002) (92)	Pig	HBD	Continuous	1. NMP (N=5) 2. CS (N=5)	39	24	Autologous blood (Yes)	Dual	Continuous	Flow	AST, ALT, GGT, bile flow, factor V, histology	AST, ALT significantly lower in NMP group, it showed a continual rise in CS. GGT lower in NMP group. Factor V was lower and showed a decrease in NMP group. Greater necrotic parenchyma in CS
Butler et al. (2002) (95)	Pig	HBD	Continuous	1. NMP (N=5)	39	72	Donor blood (Yes)	Dual	Continuous	Flow	ALT, urea, creatinine, factor V, AP, bilirubin, histology	Urea and creatinine increased; factor V remained constant. ALT slightly decreased; AP decreased more. Bilirubin remained low but increased during NMP. No overall architectural damage.
St Peter et al. (2002) (71)	Pig	DCD (WI=60min)	Continuous	1. NMP (N=4) 2. CS (N=4)	38	24	Autologous blood (Yes)	Dual	Continuous	Flow	ALT, AST, LDH, bile flow, factor V, glucose, histology	AST, ALT, LDH rose in CS group, lower in NMP. Bile flow and factor V was higher in NMP group. NMP livers had better-preserved architecture, no necrosis.
Brockmann et al (2009) (169)	Pig	HBD/DCD (40/60min)	Continuous	1. CS-HBD-5h (N=5) 2. NMP-HBD-5h (N=5) 3. CS-HBD-20h (N=7) 4. NMP-HBD-20h (N=4) 5. CS-DCD40-20h (N=4) 6. NMP-DCD-40-20h (N=4)	38	5 5 20 20 20 20	Autologous blood (Yes)	Dual	Continuous	Pressure	AST, ALT, HA, histology	No difference in AST, ALT, HA in 5h CS and NMP. CS groups had higher AST, ALT levels and more necrosis and hemorrhage compared to NMP for 20h. 40 min WIT livers with NMP showed less necrosis compared to CS, while leavers with 60min

				7. NMP-DCD60-20h (N=4)		20						WIT showed extensive apoptosis and necrosis.
o. d. Dries et al. (2013) (117)	Human	DCD/ECD (15.5 min average)	End-ischemic (CIT= 415 ± 58 min)	1. NMP (N=4)	37	6	Donor Blood (Yes)	Dual	HA: Pulsatile PV: Continuous	Pressure	ALT, AP, GGT, bile flow, bilirubin, lactate, histology	Stable ALT, GGT levels, AP decreased. Lactate rose initially but then decreased. Bile was produced throughout the perfusion period. Bile quality improved, as shown by bilirubin. Biliary GGT, LDH decreased. No biliary injury.
Boehnert et al. (2013) (215)	Pig	DCD (60 min)	End-ischemic (CIT=4h)	1. NMP (N=6) 2. CS-4h (N=6) 3. CS-12h (N=6)	38	8	Steen solution (Yes)	Dual	Continuous	Pressure	ALT, bile flow, bilirubin, bile salts, LDH, AST, histology	ALT remained low, bile was produced and necrosis was minimal during NMP. After reperfusion NMP livers had lower ALT, less necrosis and greater O2 consumption than CS. No difference in bile flow, NMP had more bilirubin, bile acid levels, less LDH and no bile duct injury.

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Table 5. *Continued*

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Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/Flow-controlled	Major Endpoints	Outcome
Schlegel et al. (2014) (189)	Rat	DCD (30/60min)	End-ischemic (CIT=4h)	1. NMP-healthy-full blood (N=10) 2. NMP-WI-full blood (N=10) 3. NMP-WI- leukocyte-depleted (N=10) 4. CS-WI (N=10) 5. CS-HOPE-WI (N=10)	37 37 37 4 4	4 4 4 4 4+1	NMP: 1) Full blood (Yes) 2) Leukocyte- and platelet-depleted blood (Yes) HOPE: UW-solution (Yes)	NMP: Dual HOPE: PV	NMP: HA: Pulsatile PV: Continuous HOPE: Continuous	NMP: Pressure HOPE: Pressure	AST, bile flow, tissue ATP, histology	Healthy NMP livers had low AST, stable bile flow and high ATP; Kupffer and endothelial cells were not activated. DCD-NMP livers had low AST but Kupffer and endothelial cells were activated. Bile flow was independent of perfusate. Higher AST, lower ATP and bile in CS group. HOPE prevented cell activation and had the lowest AST.
Nassar et al. (2015) (120)	Pig	DCD (60 min)	Continuous	1. NMP (N=15) 2. CS (N=5)	38	10 10	Whole blood (Yes)	Dual	HA: Pulsatile PV: Continuous	Pressure	AST, ALT, LDH, ALP, bile flow, histology	AST, ALT, LDH and ALP were significantly lower in NMP group. Bile flow increased during NMP and was higher than CS. NMP livers showed no or limited necrosis (<25%) in contrast to CS (>75%)
Ravikumar et al. (2016) (226)	Human	DBD/ DCD (14-31 min)	Continuous	1. NMP (N=20) 2. CS (N=40)	37	9.3 (3.5-18.5) 8.9 (4.2-11.4)	Packed RBCs in Gelofusine (Yes)	Dual	Continuous	Pressure	AST, ALP, bilirubin, INR	AST and bilirubin were significantly lower in NMP group. ALP and INR were similar between the two groups. EAD in 3 NMP livers (15%) compared to 9 CS (22.5%)
Selzner et al. (2016) (188)	Human	HBD/ DCD (21-74 min)	End-ischemic (CIT<2h)	1. NMP (N=10) 2. CS (N=30)	37	8 (5.7-9.7)	Packed RBCs and Steen solution (Yes)	Dual	Continuous	Pressure	AST, ALT, bilirubin, lactate	AST, ALT were stable during NMP, lactate decreased, HA and PV flows were stable, NMP group had lower AST,

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												ALT than CS, bilirubin levels were similar. No biliary complications in NMP group.
Banan et al. (2016) (151)	Human	DBD/ DCD (18min)	End-ischemic	1. NMP (N=7)	37	8	Donor blood (Yes)	Dual	Continuous	Flow	AST, ALT, ALP, histology	AST, ALT stabilized after 4h. Bile flow increased until hour 4 and remained stable. INR started high and then declined gradually. Hepatocyte integrity was grossly normal, minimal to complete lack of IRI
o. d. Dries et al. (2016) (153)	Rat	HBD/ DCD (30 min)	Continuous	1. NMP-HB (N=7-9) 2. NMP-DCD (N=7-9) 3. CS-HBD (N=7-9) 4. CS-DCD (N=7-9)	37	3 3 3	RBCs with Williams' medium E (Yes)	Dual	HA: Pulsatile PV: Continuous	Pressure	AST, ALT, LDH, bile flow, GGT, bilirubin	AST, ALT, LDH significantly lower in NMP livers (both groups). Bile flow lower in NMP-DCD than in CS-DCD. CS livers had lower bilirubin and higher GGT than NMP.

Table 5. *Continued*

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Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/Flow-controlled	Major End-points	Outcome
Nassar et al. (2016) (187)	Pig	DCD (60 min)	Continuous	1. NMP (N=5) 2. SNMP (N=5) 3. CS (N=5)	38.5 21 4	10 10 10	Full blood (Yes)	Dual	Continuous	Pressure	AST, ALT, LDH, bile flow, GGT, histology	AST, ALT significantly lower in NMP. LDH levels did not reach significance. Bile flow was higher and GGT levels were lower in NMP livers. Diffuse necrosis occurred in CS, less pronounced in SNMP and absent in CS. CS, SNMP livers showed loss of cellular architecture.
Bral et al. (2017) (190)	Human	DCD/DBD (18.75 min, 16-26 min)	End-ischemic (CIT= 1.58-4.97h)	1. NMP (N=10) 2. CS (N=10)	37	24	Sterofundin with packed RBCs (Yes)	Dual	Continuous	Pressure	ALT, LDH, bile flow, O_2 consumption, histology	AST, ALT rose during NMP. Bilirubin increased in all cases. Bile flow, bilirubin and AST were higher in DCD livers. Post-transplant AST, ALT, lactate and AP were similar for NMP and CS. 1 NMP liver discarded due to operator error.
Vogel et al. (2017) (123)	Human	DCD/ECD (11.3 min, 3-15min)	End-ischemic (CIT=9.5h. average)	1. NMP (N=13)	37	24	Sterofundin with packed RBCs (Yes)	Dual	Continuous	Pressure	ALT, LDH, bile flow, O_2 consumption, histology	Bile flow showed differences between livers. Started 1-2h after the beginning of NMP and lasted for the 24h-period. All livers showed evidence of O2 consumption. ALT and LDH levels showed variations between livers. Degree of steatosis was variable; all livers except one showed apoptosis or necrosis.

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Nasralla et al. (2018) (100)	Human	DCD/DBD (21 min, mean)	End-ischemic (CIT=2.1h. mean)	1. NMP (N=120) 2. CS (N=101)	37	9.13 (mean) 7.75 (mean)	Gelofusine with packed RBCs (Yes)	Dual	Continuous	Pressure	AST, discard rate, PND, EAD, hospital and ICU stay, graft and patient survival	AST was reduced by 49.4% in the NMP arm compared to CS. EAD odds were 74% lower in NMP compared to CS. No difference in hospital and ICU stay between the two arms. EAD rates were lower in NMP arm. 50% fewer discarded livers in NMP group, resulting in 20% more transplanted livers.

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Table 6. *Results Table for Subnormothermic Machine Perfusion*

Author (Year)	Species	Graft Type (WIT)	Timing	Experimental Groups (N)	Temperature (ºC)	Duration (h)	Perfusate (Oxygenated)	Perfusion Route	Flow Type	Pressure/ Flow-controlled	Major End-points	Outcome
Vairetti et al. (2007) (193)	Rat	HBD	Continuous	1. SNMP (N=6) 2. CS (N=6)	20	6	Krebs-Henseleit solution (KH) (Yes)	PV	Continuous	Flow	AST, ALT, LDH, GGT, ATP	AST, ALT, LDH and GGT were lower in the SNNP group. No difference in bile production. SNMP groups showed higher ATP levels.
Vairetti et al. (2009) (99)	Rat	HBD	Continuous	1. SNMP, lean/fat (N=4-6 each) 2. HMP4, lean/fat (N=4-6 each) 3. HMP8, lean/fat (N=4-6 each) 4. CS, lean/fat (N=4-6 each)	20 4 8	6	Krebs-Henseleit solution (KH) (Yes)	PV	Continuous	Flow	AST, LDH, bile flow, GGT, ATP, ADP	AST, LDH lower in MP groups than CS in both models (lowest in HMP4). SNMP allowed better ATP levels in both models. Bile flow in fat livers was higher in SNMP, with less GGT.
Ferrigno et al. (2011) (146)	Rat	DCD (30 min)	Continuous	1. SNMP (N=5) 2. CS, DCD (N=5) 3. CS, HBD (N=7)	20	6	Modified UW-G solution (Yes)	PV	Continuous	Flow	AST, ALT, LDH, bile production, GGT, AST and ALT in bile, ATP	Less AST, LDH in SNMP than CS, and similar to HBD. More bile in SNMP with less enzyme release (lower GGT, AST, ALT in bile). Higher ATP in SNMP, similar to HBD.
Berendsen et al. (2012) (97)	Rat	DCD (30 min)	Continuous	1. WI SNMP (N=6) 2. Fresh SNMP (N=6) 3. WI (N=4) 4. CS (N=4)	21 21 - 4	3 3 - 3	Williams Medium E (Yes)	PV	Continuous	Flow	AST, ALT, ATP, bile production	WI SNMP group had higher ALT than fresh and CS control. AST was higher in both SNMP groups. Bile flow in SNMP was lower than normal and ATP after 3h was higher in WI-SNMP compared to fresh group, higher than CS control.
Gringeri et al. (2012) (192)	Pig	DCD (60 min)	Continuous	1. SNMP (N=5) 2. CS (N=5)	20	6	Celsior solution (Yes)	Dual	Continuous	Flow	LDH, AST, lactic acid, histology	LDH, AST and lactic acid were significantly lower in SNMP group. Rare isolation necrosis in SNMP group compared to widespread necrosis in CS group.
Bruinsma et al. (2014) (96)	Human	DCD/ ECD (23-34min)	End-ischemic (CIT=473 -871 min)	1. SNMP (N=7)	21	3	Phenol-red Williams E solution (Yes)	Dual	Continuous	Flow	ALT, LDH, ALP, ATP, bile flow, bile acids	Most ALT release was limited in the first 20 min. Moderate LDH, ALP release was observed. 3.7-fold increase in ATP (high-ATP

						DOI:10.14753/SE.2024.2895						livers had low ALT, LDH, ALP). Bile flow started in first 30min, then decreased together with bile salts. Normal hepatocyte morphology, no injury to sinusoidal epithelium
Fontes et al. (2015) (191)	Pig	HBD	End-ischemic (CIT=9 h)	1. SNMP (N=6) 2. CS (N=6)	21	7.47 (mean)	Cell-free HBOC solution (Yes)	Dual	HA: Pulsatile PV: Continuous	Flow	AST, LDH, lactate, oxygen, histology	O_2 delivery was 8-fold higher than consumption in SNMP. Much lower AST, ALT and higher bile flow in SNMP group. No to mild IR injury in SNMP, mild to moderate in CS group.