Do not leave the Liver hanging:
redesigning the Organ Chamber to enable long term ex situ Liver Perfusion

P.C. Groen

DO NOT LEAVE THE LIVER HANGING:

REDESIGNING THE ORGAN CHAMBER TO ENABLE LONG TERM EX SITU LIVER PERFUSION

by

Puck Caroline Groen

Student number: 4470044

May 10, 2023

Thesis in partial fulfilment of the requirements for the joint degree of Master of Science in

Technical Medicine

Leiden University, Delft University of Technology & Erasmus University Rotterdam

Master thesis project (TM30004, 35 ECTS)
Erasmus MC
Department of Transplantation Surgery
July 2022 – May 2023

Chair and medical supervisor:	Dr. Jeroen de Jonge, MD	Erasmus MC
Technical supervisors:	Prof. dr. Arjen Amelink	TNO Delft
	Michiel Oderwald, MSc	TNO Delft
Independent committee member:	Prof. dr. Marcel Ottens	TU Delft

Universiteit Leiden

TU Delft Delft University of Technology

ERASMUS UNIVERSITEIT ROTTERDAM

Abbreviations

ALT	-	Alanine aminotransferease
AST	-	Aspartate aminotransferease
GGT	-	Gamma-glutamyl transferase
ATP	-	Adenosine triphosphate
CIT	-	Cold ischemia time
COR	-	Controlled oxygenated rewarming
DBD	-	Donation after brain death
DCD	-	Donation after circulatory death
(D)HOPE	-	(dual) Hypothermic oxygenated machine perfusion
DoB	-	Delta over baseline
EAD	-	Early allograft dysfunction
ECD	-	Extended criteria donor
FDG	-	Fluorodeoxyglucose
(f)WIT	-	(functional) warm ischemia time
HA	-	Hepatic artery
HMP	-	Hypothermic machine perfusion
ICG	-	Indocyanine green
ICU	-	Intensive care unit
IRI	-	Ischemia reperfusion injury
IVC	-	Inferior vena cava
LiMAx	-	Liver maximum capacity
LSCI	-	Laser speckle contrast imaging
MP	-	Machine perfusion
Na	-	Sodium
NMP	-	Normothermic machine perfusion
OR	-	Operating room
PE	-	Polyethylene
PET-CT	-	Positron emission tomography combined with computed tomography
PNF	-	Primary non function
PV	-	Portal vein
ROS	-	Reactive oxygen species
SBP	-	Systolic blood pressure
SCS	-	Static cold storage
SNMP	-	Subnormothermic machine perfusion

Table of contents

Preface

This report is the result of my master thesis of the MSc Technical Medicine with specialization in the track Imaging and Intervention. With this report, my seven years of studying at Delft University of Technology, Leiden University and Erasmus University has come to an end. During my second internship of the master, I came into contact with the field of liver transplantation and especially with the field of organ perfusion through Dr. Jeroen de Jonge. This field immediately caught my attention, as it is relatively new and there is so much more to discover. And most importantly: there is so much more to gain. This graduation project continued where I left after my internship, and I cannot wait to further develop myself in the field of liver transplantation and perfusion as an Organ Perfusionist in the Erasmus MC.

Naturally, I could not have completed this thesis without the help of others. I would like to express my gratitude and appreciation. Especially, I want to thank Jorke for his expertise, his critical eye and for his help during the long perfusions, which mostly happened during the weekends and at night. Furthermore, I would like to thank all liver perfusionists from the Erasmus MC: Stef, Femke, Efrayim, Roberto, Ivo and Fenna, for teaching me the basics of organ perfusion and helping me with my perfusion experiments.

I would like to thank my graduation committee, Jeroen, Michiel and Arjen, for their valuable insights, expertise, critical eye and motivating attitude. Jeroen, your enthusiasm for liver transplantation and liver perfusion worked contagious on me. I enjoyed our discussions on possible solutions, and I loved that in this project, I could 'knutsel' an organ chamber with everything I could think of. I look forward to the upcoming years as an Organ Perfusionist and PhD candidate in your team. Michiel and Arjen, your insights from the business and developing perspective proved to be very valuable, especially in the beginning of this thesis. From you I learned to start simple: what do we need and why. From there on, this project grew into wat it is now, and hopefully in the future we can keep working together on improving organ perfusion and organ evaluation.

Finally, I would like to thank my friends, family and especially my roommates for their support and willingness to listen to my 'thesis talk' for the past year.

Enjoy reading,

Puck Groen
Rotterdam, May 2023

Summary

Introduction

The only curative and durable treatment option for patients suffering from end-stage liver disease is liver transplantation. However, there is a shortage of donor grafts of adequate quality, which is a major cause of wait list mortality. *Ex situ* liver resuscitation, reconditioning or regeneration of sub-optimal donor grafts can potentially enlarge the number of available donor grafts. However, this can only be achieved using long term normothermic machine perfusion (NMP). The current clinically relevant perfusion devices do not fully mimic the *in situ* environment of the liver, which results in gravity induced pressure injuries and poor peripheral perfusion. The aim of this thesis is to create an organ chamber that is similar to the *in situ* situation, capable of preventing gravity induced pressure injuries and improving peripheral perfusion.

Materials & Methods

Two prototypes were fabricated using materials bought at the DIY store. Perfusions were performed with porcine (N=3) and human research livers (N=2) using an adapted Liver Assist (XVIVO abdominal) perfusion system. Both prototypes were part of a closed loop perfusion system and in both human research livers all three main vessels were cannulated. Temperature control and perfusion stabilization was tested during perfusion. The water in the reservoir was heated from 20°C to 37 °C during the first hour, followed by NMP during which the temperature was kept at 37 °C. The influence of the amount of water in the water reservoir on the perfusion parameters was tested by altering the height of the water level. Perfusate samples from the human research livers were taken at set time points.

Results

Both prototypes were capable of controlled rewarming of the liver to 37 °C during the first hour of perfusion. After reaching 37 °C the temperature remained stable. During perfusion with both prototypes, perfusion parameters could be set to the desired target values and the flows remained stable. The second prototype was used to test the effect of different water levels in the water reservoir on the perfusion parameters. With 7 centimeters of water in the reservoir, the portal vein flow was 940 ml/min. With 12 centimeters of water, the portal vein flow started to decrease until it reached a flow of 660 ml/min with the maximum of 23 centimeters of water. The hepatic artery flow was not influenced by the varying water level. The inferior vena cava pressure slowly decreased from 2 mmHg with 7 centimeters of water, to -3 mmHg with 23 centimeters of water. Both perfusions showed an increase in injury markers during perfusion.

Conclusion

In this thesis, a new approach to the organ chamber was explored, to prevent gravity induced pressure injuries and improve peripheral perfusion. Both prototypes were capable of maintaining stable perfusion parameters. The amount of water surrounding the liver influences the flow of the portal vein and the pressure of the inferior vena cava. In the future, this effect could be used to mimic the function of the diaphragm and improve the peripheral perfusion. The next step is to quantify and visualize peripheral perfusion and improve the outcomes of long term liver perfusion.

1. Introduction

1.1 Liver transplantation

Liver transplantation is the only curative treatment option for patients suffering from end-stage liver disease. However, the number of transplantable organs is not able to meet the demand. The incidence of liver diseases, for which liver transplantation is required, is increasing[1,2]. Simultaneously, the quality of donor grafts is decreasing, as donors are of increasing age and have a higher BMI[3,4]. The gap between the number of suitable donor grafts and the demand is a major cause for wait list mortality. In 2021 in the Netherlands, 240 patients were removed from the waiting list[5]. Of these 240 patients, 30 died waiting for a liver, and 8 got removed from the waiting list due to deterioration of their condition, equaling a wait list removal of 16%.

1.2 Organ donation – donation after brain death versus donation after circulatory death

In the Netherlands, there are two possibilities to donate organs after death: donation after brain death (DBD) and donation after circulatory death (DCD). DBD donors have irreversible loss of function of the brain and brain stem and are declared brain dead based on neurological criteria according to Dutch law. The donor is brought to the operating room (OR) while still on life support and at this moment, the organs are still supplied with oxygenated blood. The circulation is stopped by flushing all organs simultaneously with a cold preservation solution, starting the cold ischemia time (CIT) and life support is discontinued. An overview of important events during a DBD procedure is shown in Figure 1A.

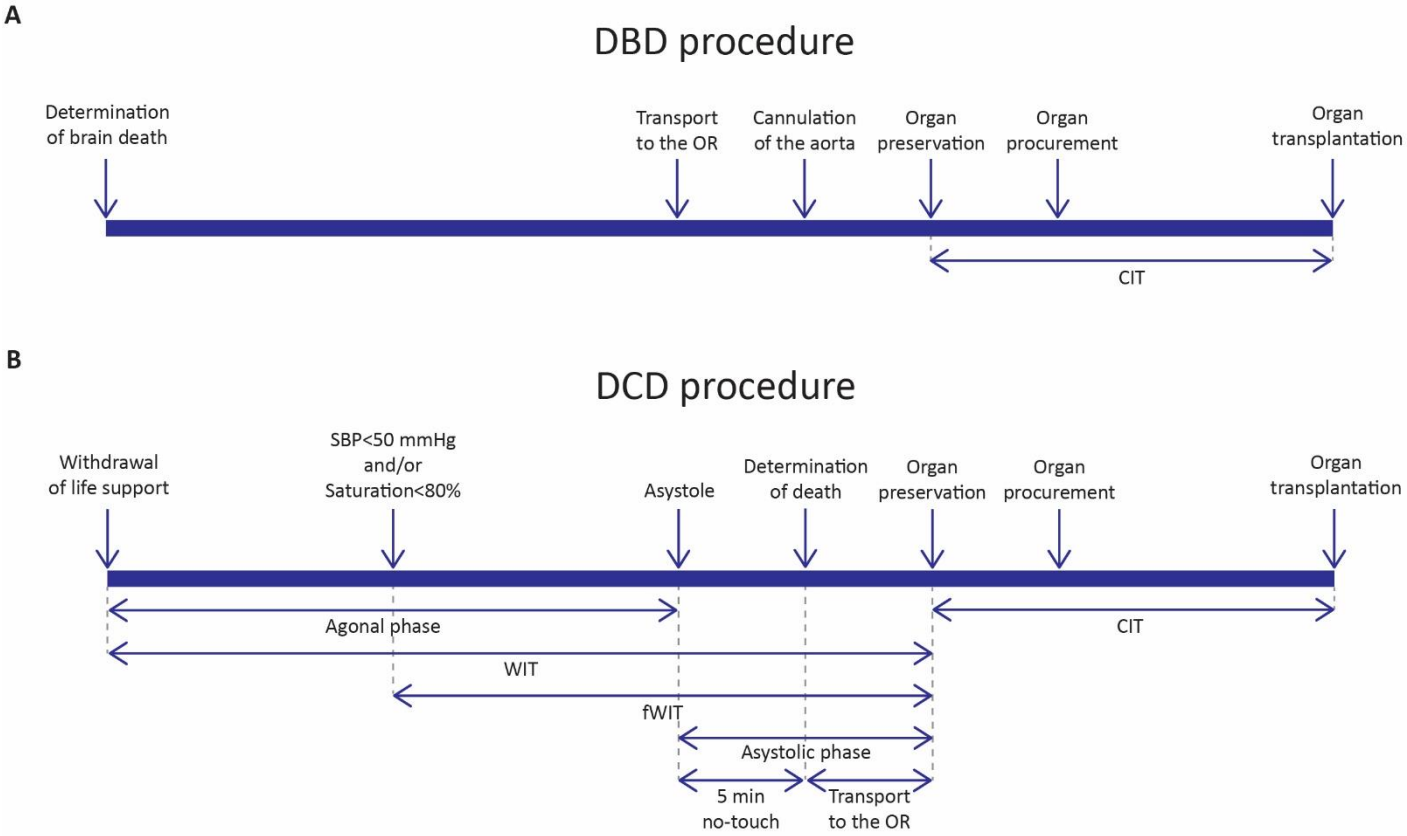


Figure 1. *An overview of important events during a DBD and DCD procedure.*

In DCD donors, brain death cannot be determined, but the prognosis of the donor is infaust. Withdrawal of life supporting treatment will usually lead to hypoxia, subsequent circulatory arrest, and death. The DCD procedure is dictated by Dutch law and is shown in Figure 1B. The withdrawal of life support, the switch-off, takes place on the intensive care unit (ICU). The warm ischemia time (WIT) starts after switch-off, and during this phase the donor is closely monitored, but no medical treatment is given. Circulatory arrest can occur within minutes, but also within hours after withdrawal. When the saturation of the donor drops below 80% and/or the mean arterial pressure drops below 50 mmHg, the functional warm ischemia time (fWIT) starts. For organ quality, and for the liver specifically, the WIT should not be longer than 1 hour, and the fWIT should not exceed 30 minutes. During this period the organs are at body temperature, but not supplied with enough oxygen, causing ischemic damage. A mandatory 5-minute no-touch period starts when the donor goes into circulatory arrest, after which death is declared. The donor is then transferred to the OR, where the organs are perfused with a cold preservation solution as soon as possible, ending the (f)WIT and starting the CIT. CIT has been regarded as a donor-related risk factor for graft failure in both DBD and DCD and the CIT of the liver should not be stretched beyond 12 hours[6,7]. The periods of WIT and fWIT, visualized in Figure 1B, are the important differences between DBD and DCD organs, and the reason for considering DCD organs inferior.

The shortage of donor livers, in combination with changes in organ donor demographics, such as increasing age and higher BMI, has led to the utilization of livers of suboptimal quality and livers from extended criteria donors (ECD)[8,9]. These livers would have been rejected for transplantation because of donor characteristics, such as age, obesity, alcohol abuse, or a long period on the intensive care unit with vasopressors, or graft pathology, such as steatosis, elevated liver enzymes, or a history of liver trauma (Figure 2)[10-12].

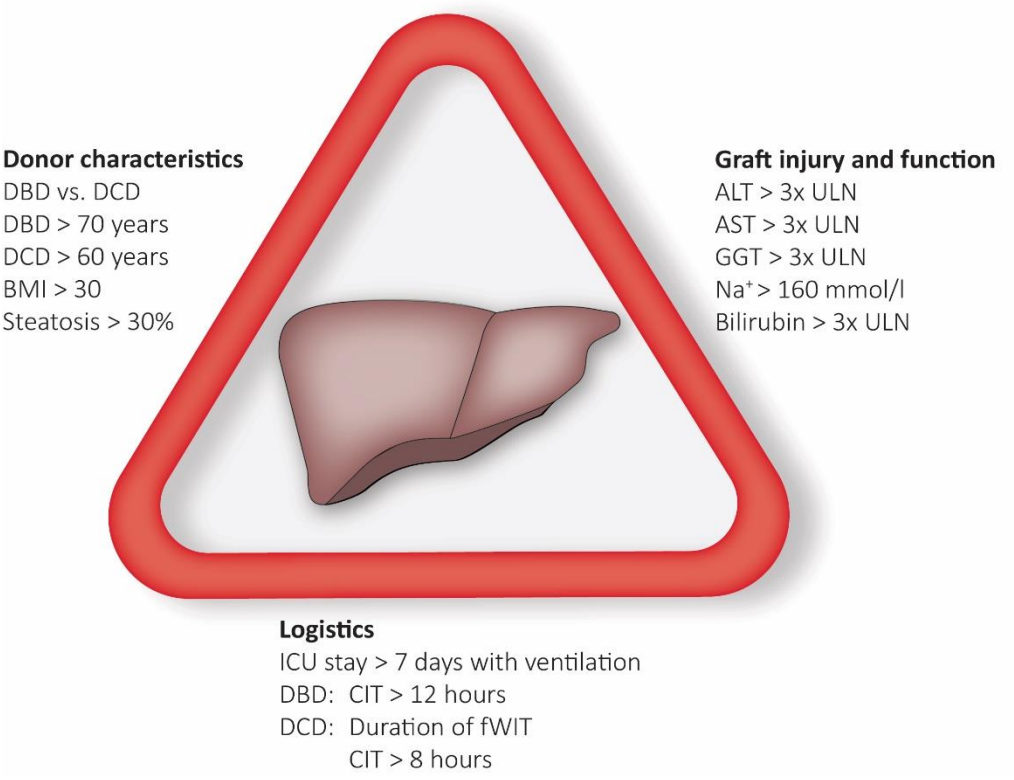


Figure 2. *Criteria for an ECD liver. ALT, alanine aminotransferase; ULN, upper limit of normal; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; Na+, sodium.*

ECD livers bring an increased risk of developing early allograft dysfunction (EAD), primary non-function (PNF), and post-transplant cholangiopathy[10,13-19]. It is difficult to predict the onset of these complications upfront considering risk factors present, without measuring the quality and viability of the liver. For recipient safety reasons, high risk donor livers are often discarded for transplantation[20]. In the Netherlands, 271 donor procedures were performed in 2021, however, only 167 livers were transplanted, equaling an overall discard rate of 38%, and a discard rate of even 56% if only DCD donors are considered[5].

1.3 The traditional way of preserving donor organs – keeping it cool

The standard procedure of organ preservation after retrieval includes flushing the organ with a cold preservation solution and placing it on ice during transportation to the transplant center. This method of preservation is called static cold storage (SCS). SCS relies on low temperatures and the preservation solution to decrease the cellular metabolism. Although the metabolism in the organ is significantly decreased, there is still anaerobic activity, which leads to an array of intracellular changes[21]. During ischemia succinate accumulates, and after reperfusion with oxygenated blood, succinate is re-oxidized. Re-oxidation of succinate leads to reactive oxygen species (ROS) formation by reverse electron transport at mitochondrial complex I[22]. This aggravates the injury of the organ and leads to a cascade of damage. This damage is called ischemia-reperfusion injury (IRI), and it is the result of several processes, including mitochondrial damage, subsequent adenosine triphosphate (ATP) depletion, generation of ROS, apoptosis and necrosis, inflammatory cytokine release, innate immune activation, microcirculatory failure, and platelet aggregation[22-24]. IRI can result in EAD, PNF, reduced graft survival, and post-transplant cholangiopathy. SCS is a relatively easy method to preserve organs *ex situ.* However, a considerable drawback is that SCS cannot improve the quality of the organ, due to the build-up of the aforementioned intracellular changes. Therefore, SCS preservation of the liver has a maximum duration of approximately 12 hours for DBD, and a maximum of 8 hours for DCD. Another drawback of SCS is that, because of the reduced metabolism, options to assess the quality and viability of the organ are limited.

1.4 Perfusing donor organs – preserving livers in a dynamic environment

To overcome the limitations imposed by SCS, numerous methods for organ preservation have been developed to optimize and test the conditions of organs prior to transplantation. *Ex situ* machine perfusion (MP) is a dynamic preservation method that may better protect organs from IRI and may improve outcomes after organ transplantation[25,26]. The concept behind liver MP is creating a circuit, using a pump, to circulate a preservation solution through the vasculature of the liver[27,28]. The liver has two supplying blood vessels: the portal vein (PV) and the hepatic artery (HA). The PV transports nutrient rich, oxygen poor blood from the gastrointestinal tract to the liver and provides approximately 75% of the total blood flow to the liver and the HA transports nutrient poor, but oxygen rich blood from the aorta to the liver. Blood flows from the liver through the hepatic veins to the inferior vena cava (IVC). During liver perfusion, the PV is cannulated, and depending on the perfusion strategy and perfusion machine, the HA, IVC and bile duct are also cannulated. The different strategies for MP can be broadly subdivided based on the temperature at which the perfusion is performed: hypothermic (4-12 °C), sub-normothermic (12-34 °C) and normothermic (35-37 °C) MP.

Hypothermic machine perfusion (HMP) can be non-oxygenated or oxygenated. Non-oxygenated HMP provides a continuous flow of metabolic substrates and antioxidants, but no oxygen, and flushes

cytokines, proteins, and toxins from the liver. During oxygenated HMP, the liver is provided with plenty of oxygen either through only the PV, which is called hypothermic oxygenated machine perfusion (HOPE), or though both the PV and the HA, called dual hypothermic oxygenated machine perfusion (DHOPE). The goal of (D)HOPE is to restore mitochondrial function and intrahepatic ATP levels. Providing oxygen during (D)HOPE prevents the accumulation of succinate and, therefore, formation of ROS and oxygen restores the electron transport across the mitochondrial membrane. A large randomized controlled trial performed by Van Rijn *et al.* showed that DCD livers that received DHOPE after a period of SCS had a lower incidence of nonanastomotic biliary strictures compared to livers that only received SCS[25]. Performing non-oxygenated HMP or (D)HOPE is relatively safe and simple, as the lowered temperatures can protect the organ when the perfusion device malfunctions. However, at these temperatures, the liver has a reduced metabolism. Therefore, the organ's functionality cannot accurately be assessed during HMP.

Normothermic machine perfusion (NMP), on the other hand, preserves the liver at body temperature. The goal of NMP is to keep the liver in a near physiological state, which creates an optimal environment in which the liver can resume metabolic activity. This does mean that the liver is consuming oxygen and nutrients, which will have to be actively replenished, while metabolic waste products, such as CO_2, are removed from the perfusate. Increasing the metabolic rate to a near-physiological state allows for assessment of the function and viability of the liver and provides objective information to aid in the decision whether the liver can safely be transplanted or not. This can lead to an increased use of ECD organs, reduce the relatively high discard rate of donor organs and ultimately, reduce wait list mortality. A recent European randomized trial in liver transplantation performed by Nasralla *et al.* demonstrated safety and potential benefits of NMP over SCS, with 50% fewer rejected livers in the NMP group, resulting in 20% more transplants compared to SCS[29].

An alternative approach is subnormothermic machine perfusion (SNMP), where the temperature is subphysiological and typically lays between 25 °C and 34 °C[30]. At these temperatures, the liver graft is metabolically less active than during NMP, but still provides the possibility for viability assessment. A variation of SNMP is controlled oxygenated rewarming (COR), during which the temperatures are gradually increased. Minor *et al.* performed COR from 10 °C to 20 °C and showed lower markers of hepatocellular injury after transplantation and enhanced graft function after COR compared to SCS[31].

1.5 Commercially available perfusion machines

There are different perfusion machines on the market, and Table 1 provides an overview of CE marked machines and their perfusion strategies.

Table 1. *An overview of CE marked perfusion machines.*

Machine	Manufacturer	Perfusion strategy
Aferetica PerLife[32]	Aferetica, Bologna, Italy	HMP, SNMP, NMP
Liver Assist[33]	XVIVO, Goteborg, Sweden	HMP, SNMP, NMP
OrganOx Metra[34]	OrganOx Limited, Oxford, UK	NMP
Organ Care System Liver[35,36]	TransMedics, Andover, USA	NMP
VitaSmart Machine Perfusion System[37]	Brigde to Life Europe, London, UK	HMP

In the Erasmus MC, the Liver Assist and the OrganOx Metra are used in clinical practice, and therefore these machines will be discussed in more detail. Both machines require sterile plastic disposables to perform liver perfusion.

The Liver Assist can be used for DHOPE, SNMP and NMP (Figure 3A). The disposable set of this machine has an intended use of 6 hours. It is an open circuit, with the IVC draining freely into the reservoir. The liver rests in a plastic net in the reservoir and the liver touches the perfusion solution (Figure 3B). The PV and the HA are cannulated, as is the bile duct during NMP. When using this machine, it is required to manually change pressure and temperature settings as is changing the oxygen and air flow. The OrganOx Metra performs NMP, and the disposable set has an intended use of 24 hours Figure 3C). This machine is designed to perform each perfusion in a reproducible manner, which is achieved by maintaining the same target pressures and target flows during every perfusion. The settings of this machine cannot be changed manually. This machine has a closed circuit, thus the HA, PV and IVC are cannulated, as is the bile duct. The liver rests in a bowl with a silicon mat, and leaked perfusate is pumped back to the reservoir using a roller pump (Figure 3D).

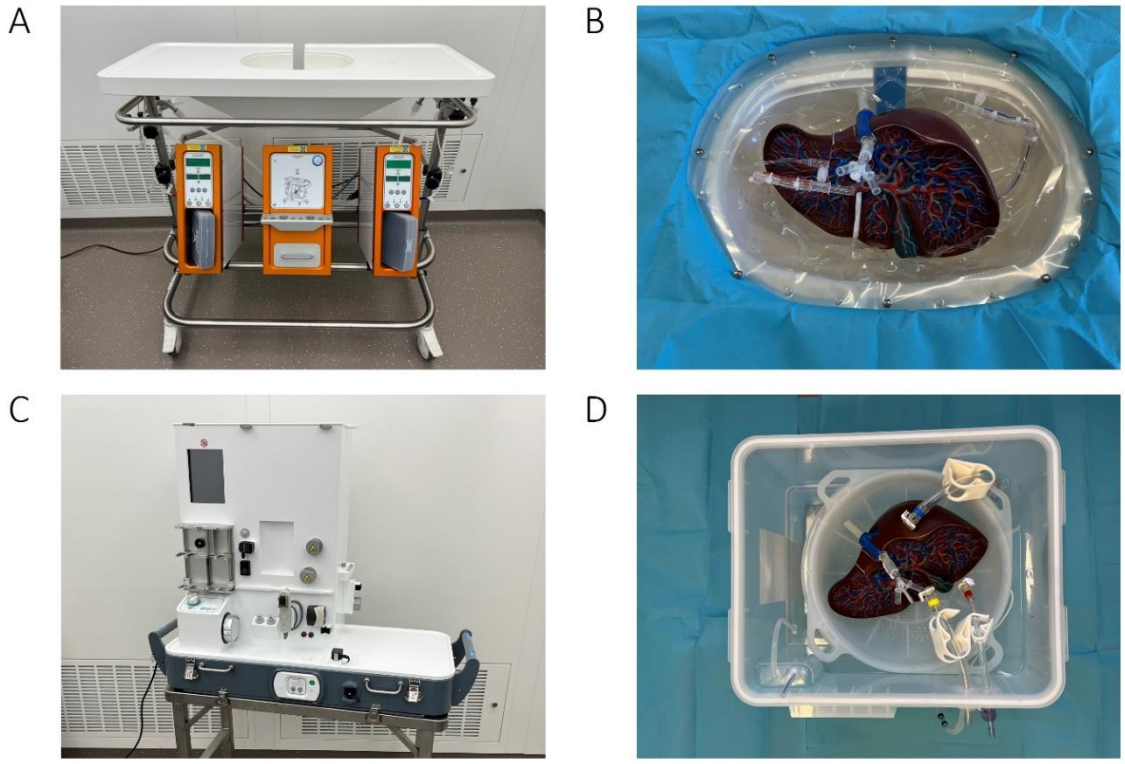


Figure 3. *The Liver Assist (A), the organ chamber of the Liver Assist (B), the OrganOx Metra (C) and the organ chamber of the OrganOx Metra (D).*

1.6 How to perfuse a liver? Current clinically used perfusion protocols

In the Netherlands, *ex situ* MP of the liver is performed in the recipient center, a back-to-base approach. The different liver preservation methods and their maximum preservation duration are shown in Figure 4 and these methods are used for different indications.

According to Dutch national protocol, every DCD liver is put on (D)HOPE in the recipient center. For assessing ECD livers prior to transplantation, Groningen University has developed an extended perfusion protocol[38,39]. This protocol includes 1 hour of DHOPE, followed by 1 hour of COR from 20 °C to 37 °C, and 2.5 hours of NMP. After 2.5 hours of NMP, predetermined viability criteria need to be met to allow for transplantation. If these criteria are met and thus the liver is considered viable, the

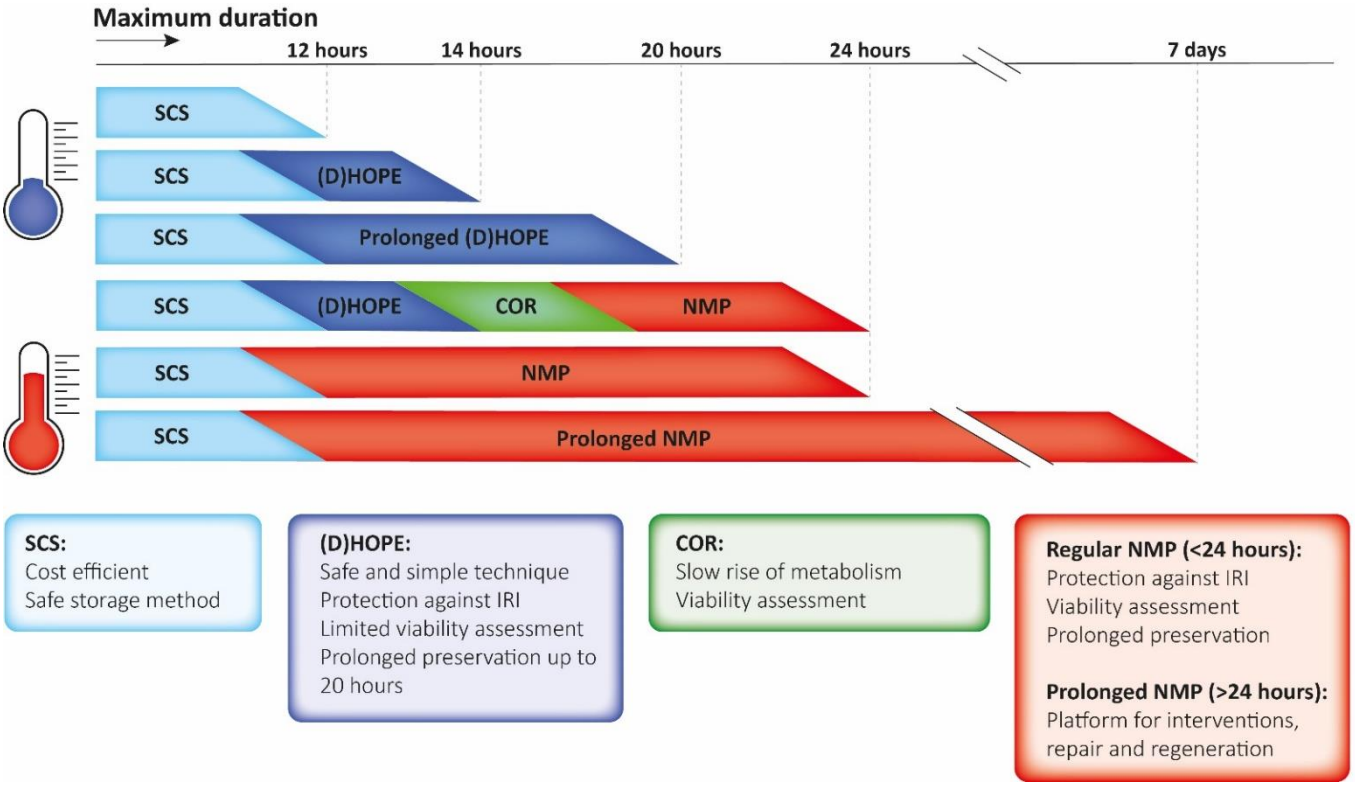


Figure 4. *An overview of the different liver preservation methods and the current reported maximum preservation duration. Traditional SCS is safe up to 12 hours. (D)HOPE is typically performed after SCS for 2 hours. Prolonged preservation with DHOPE has been reported for up to 20 hours. "Regular" NMP is possible up to 24 hours. (D)HOPE can be combined with COR and NMP. Prolonged NMP enables graft preservation up to one week and maybe even longer.*

perfusion is continued until the surgical team on the OR is ready to implant the liver. NMP only is performed for ECD DBD livers, and for logistical reasons: the preservation time of the graft can be prolonged with NMP. In the Erasmus MC, both the DHOPE and the DHOPE-COR-NMP protocol are performed on the Liver Assist, and NMP only is performed on the OrganOx Metra.

1.7 Are we there yet? Current limitations

Publications from different groups have already shown the added benefit of MP in effort to increase organ utilization and decrease wait list mortality. The field of MP is relatively new but has enormous potential to change the entire field of organ transplantation. MP does not only provide opportunities to better preserve or monitor organs, but it can also create opportunities for *ex situ* repair or regeneration on the pump.

Prolonged DHOPE research is being performed to extend the preservation time of livers, which could provide more time for allocation and transplantation logistics. These processes are now limited by the maximum CIT of either 8 or 12 hours[40]. However, to create opportunities for liver repair and regeneration, prolonged NMP (beyond 24 hours) is required.

Prolonged NMP requires different machines than the current CE-marked and commercially available machines. These machines are made to perfuse the liver for a relatively short period of time, and therefore, do not include other organ systems. They should, for example, include a dialysis unit for the removal of waste products, mimicking the function of the kidney, automated hormone regulation, mimicking the function of the pancreas and movement of the liver, mimicking the function of the diaphragm[41].

In the human body, the liver is attached to the diaphragm and moves with breathing. Therefore, *in situ,* the liver is never statically resting on any structures in the abdomen. In addition, movement of the diaphragm also creates pressure changes in the abdominal cavity. When inhaling, the abdominal cavity decreases, creating a higher pressure on the liver. The liver is squeezed, and blood flows from the periphery to the liver veins and subsequently to the IVC. When exhaling, the pressure in the abdominal cavity drops and the liver is filled with blood which is distributed to the periphery. These pressure differences influence the circulation of the liver, creating a sponge like effect. However, during *ex situ* perfusion, the liver is inverted and resting on a hard surface, not subjected to any pressure differences in the environment. During perfusion pressure injuries will occur on the surface of the liver over time. An example of pressure injuries inflicted by the silicon basket of the OrganOx Metra after 10 hours of NMP is shown Figure 5A and Figure 5B shows the imprint on the liver of the plastic net of the Liver Assist. These findings are supported by literature. The group of Liu *et al.* presented an institutional developed perfusion device and developed a soft biocompatible resting mattress for the liver to be placed on[42]. After 86 hours of perfusion, the liver had severe contact injury and necrosis upon histological analysis. Comparable to their results, Eshmuminov *et al.* reported macroscopic pressure necrosis and impaired circulation shown with fluorescence imaging at the contact area of the liver with the surface of the organ chamber[43].

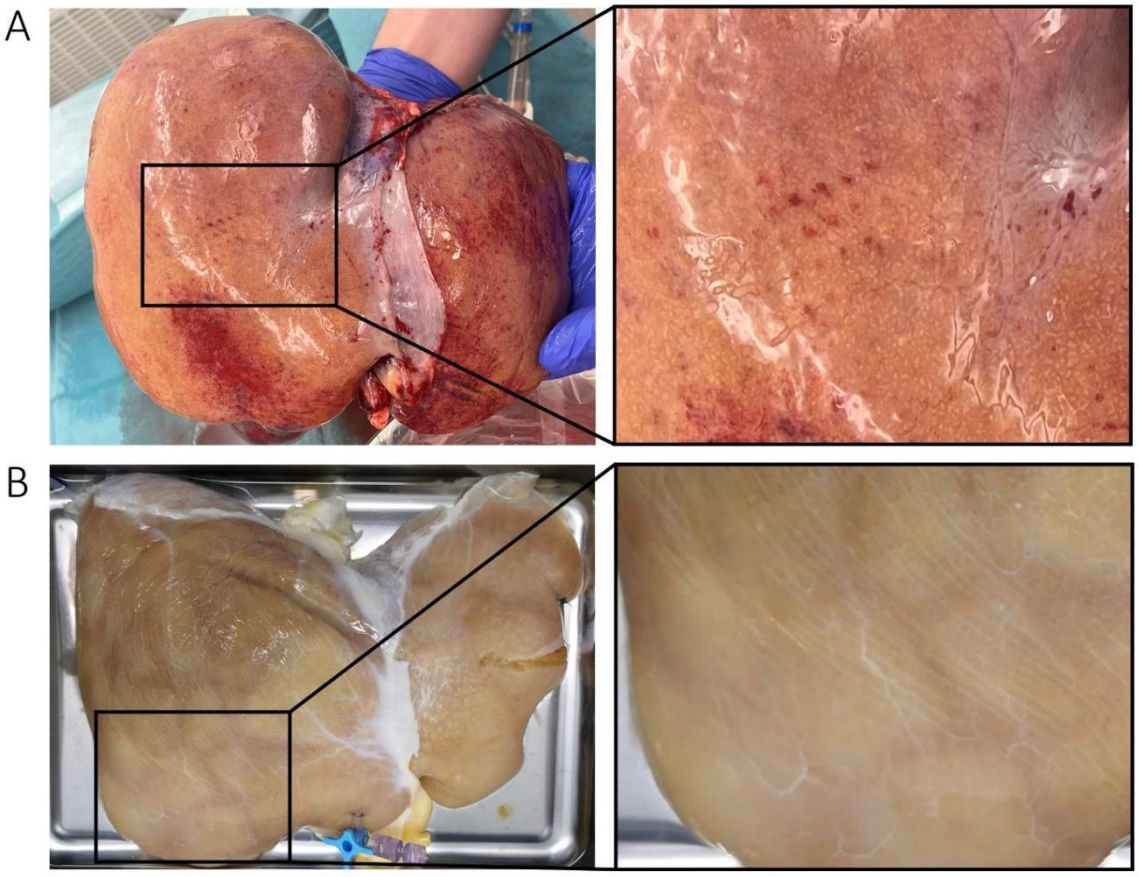


Figure 5. *Pressure injuries inflicted on the liver by the organ chambers of the OrganOx Metra (A) and the Liver Assist (B).*

These findings illustrate that not only near physiological perfusion parameters are required during *ex situ* perfusion (e.g., temperature, flow, and pressure), but that the liver also needs to be kept in a near physiological environment.

1.8 Do not leave the liver hanging – requirements for the organ chamber

A near physiological environment can be achieved by placing the liver in a specially build organ chamber, which is designed to prevent pressure injuries and mimic the sponge like effect of the diaphragm. In addition, this organ chamber should also be able to provide a stable temperature regulated environment. To be able to perform prolonged NMP, the reduction of pressure injuries, improving peripheral perfusion and providing a stable temperature regulated environment are important steps.

Interesting options for creating this physiological environment are moving the liver and submerging the liver in a fluid. The approach of the Zurich group after their experience with the pressure injuries, was introducing movement of the liver[43,44]. The organ chamber consisted of a silicon mat, beneath which an inflatable balloon was placed. The balloon was inflated and deflated 15 times per minute, mimicking the movement of the diaphragm while breathing. After 7-days of perfusion, a positron emission tomography combined with computed tomography (PET-CT) was performed, which showed no pressure injuries on the contact areas. The same group that developed the failed biocompatible mattress, looked into surrounding the liver with fluid[45]. The liver was submerged in perfusate, with the IVC draining freely into the reservoir, and no pressure injuries were seen. In this set up the perfusate served as a cushion, providing homogenous upward pressure, and it provided uniform warming. Moreover, the liver parenchyma was kept moist by the surrounding fluid. These experiences seem very promising, and therefore movement of the liver or submerging the liver in a fluid are included in the requirements for the organ chamber. Besides preventing pressure injuries, the organ chamber should also improve peripheral perfusion. The experience from the clinic shows good central perfusion of the liver, but the perfusion in the periphery of the liver stays behind. As described before, the movement of the diaphragm plays an important role in the inflow and outflow of blood. By varying the pressure in the surrounding environment of the liver, the pressure differences in the abdomen can be mimicked. Last, the liver must be in a temperature-controlled environment, which can be sealed. It should be possible to control the temperature settings of the environment and the perfusate and prevent the liver from drying out. In addition, more requirements on size, weight, materials, and sterility were drafted and can be found in the supplementary information. The organ chamber design was split into three different stages: the pilot prototype, the prototype, and the clinical prototype. The pilot prototype is the first step and the step that is completed during this master thesis. Creating the three different stages of the prototype makes it possible to make a distinction which requirements need to be met in the different stages of the designing process.

Based on the previous stated requirements, ten concepts for the organ chamber were drafted and sketches of these concepts can be found in the supplementary information. All concepts included a sealable organ chamber, for environment control, and a method for preventing pressure injuries. Improving peripheral perfusion was not possible in all concepts, and the final design choice was based on this possibility. Two concepts, concept 7 and 8, were considered the most promising, and fulfilled all requirements: controllable environment, preventing pressure injuries and improving peripheral perfusion. The choice was made to develop both concepts into prototypes. Concept 7, from now on concept 1, consisted of an inner bag for the liver to be placed in, and an outer bag that can be filled with fluid, acting as a cushion for the liver and it provides the possibility to control the temperature around the liver. Concept 8, from now in concept 2, consisted of a bag for the liver to be placed in, attached to the bottom of a container. The container can be filled with a fluid to submerge

the liver. Both concepts are based on a closed perfusion circuit, to provide the possibility to measure the pressure in the IVC.

The aim of this master thesis will be redesigning the organ chamber to reduce pressure injuries and improve peripheral perfusion, while still maintaining a stable perfusion and perfusion environment. The focus will be on preventing the liver to lay statically on a hard surface by surrounding the liver with fluid.

2. Materials and methods

2.1 Organ procurement

In this thesis, both porcine and human livers were used. Porcine livers (N=3) were retrieved from the slaughterhouse. Animals were terminated with the standardized procedure of sudden induction of brain hemorrhage, followed by exsanguination. The liver was removed en-bloc with the heart, lungs, pancreas, and spleen. After identifying the PV and HA, the PV was cannulated and the liver was flushed with 5 L NaCl 0.9% (Baxter, USA) with 25.000 IU of heparin (Leo Pharma, Denmark), providing 500 ml to the artery. Next, 2 L of cold preservation solution (Belzer University of Wisconsin (UW) solution; Bridge to Life Ltd., United Kingdom) was given through the PV. SCS was used to store and transport the livers. The porcine livers were first used for DHOPE experiments.

Additionally, two human research livers were included. The first human liver was from a DCD donor and was declined for transplantation by all centers within the Eurotransplant area. Research consent for transplantation directed research on the organ was given by the next of kin. This liver was first used for a DHOPE-COR-NMP experiment, following the protocol of Van Leeuwen *et al.*[38,46]. The second human liver, from an extended criteria DBD donor, was accepted for transplantation, but was declined after viability assessment during NMP following the OrganOx protocol. Research consent was provided by the next of kin. Both livers were procured by regional organ procurement teams using the standard procurement protocol of *in situ* cold perfusion with 6–10 L of cold preservation solution (Belzer University of Wisconsin (UW) solution; Bridge to Life Ltd., United Kingdom), containing 300 IU/kg of heparin (Leo Pharma, Denmark). The livers were stored and transported using SCS.

2.2 Prototype development

Prototypes were fabricated from vacuum storage bags of different sizes, compatible with a vacuum cleaner (Vacuum storage bag; Vacuumzakkenspecialist, the Netherlands). These bags are made from polyethylene (PE) and can be closed with a zip-lock. As these bags are made for vacuum storage, the bags were air and watertight. To adapt the size of the bags, the plastic was melted together with a simple iron or a hair straightener. Pattex Superglue plastics (Henkel, Dusseldorf, Germany), Loctite Super Glue 3 all plastics (MEM Bauchemie GmbH, Germany) or Roxolid Aktiv-X (MEM Bauchemie GmbH, Germany) were used to adhere smaller pieces of PE together. 3/8-inch tubing connectors (PE doorvoer slangtule; AquastoreXL, the Netherlands) were glued into the plastic bag.

The seams of the prototype were tested for air and water tightness after assembly. In order to find leaks, the prototypes were filled with air or water before they were submersed in water. Repairs were performed if leaks were found, and leak tests were repeated until no leaks occurred.

2.2.1 Prototype 1

Prototype 1 was based on concept 7 (supplementary information). A schematic overview of concept 7 is given in Figure 6. The prototype consisted of two bags, one for the liver to be placed in, and one for fluid that surrounds the liver. The perfusion system was a closed loop system: the PV, HA and IVC were cannulated.

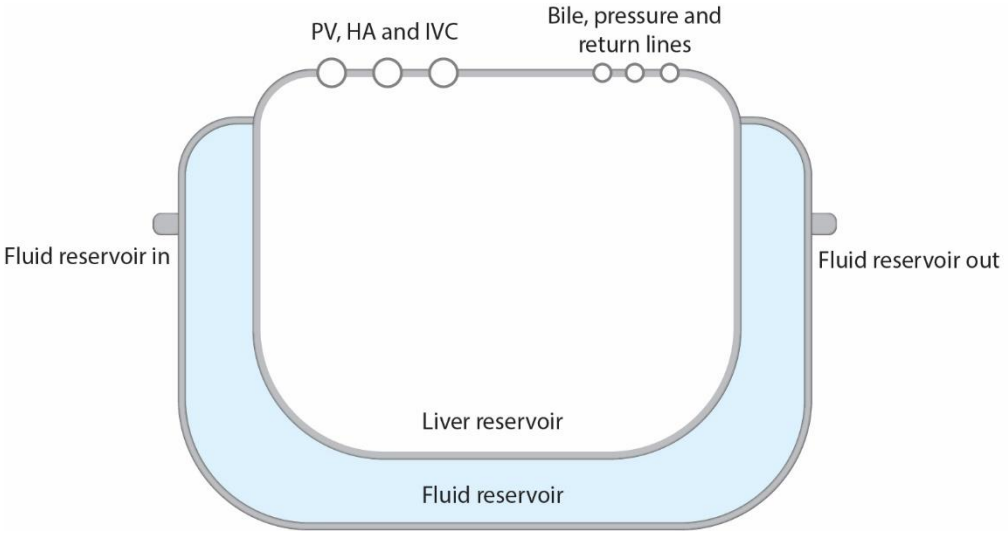


Figure 6. *A schematic overview of prototype 1.*

2.2.2 Prototype 2

Prototype 2 was developed based on concept 8 (supplementary information). It consisted of an inner bag for the liver to be placed in, and this bag was placed into a container that can contain fluid. Figure 7 provides a schematic overview of the prototype. This was a closed loop perfusion system: all three vessels are cannulated.

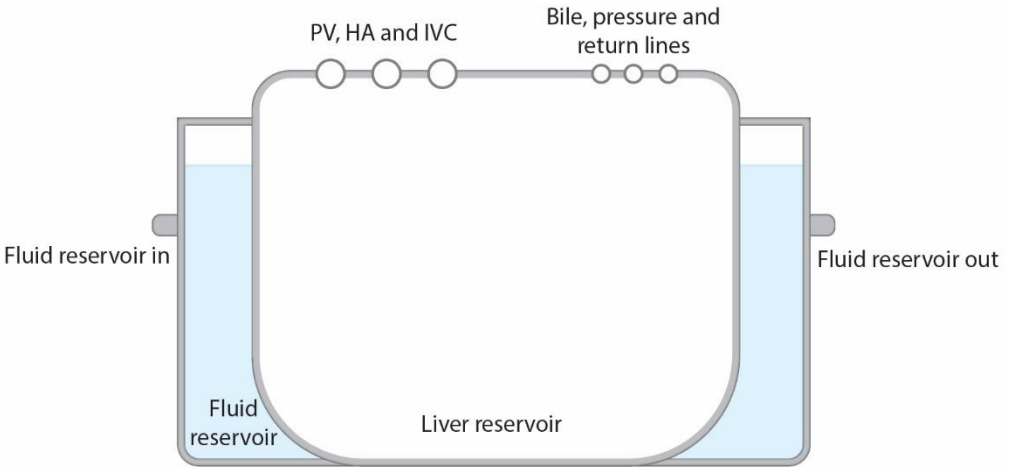


Figure 7. *A schematic overview of prototype 2.*

2.3 Connecting the prototypes to a simple perfusion circuit

The prototypes were connected to a roller pump (Masterflex; Metrohm, Switserland) and a reservoir for the perfusate (XVIVO abdominal, the Netherlands). A return line was created with 1/4-inch tubing and a Y-shaped tubing connector at the end (XVIVO, the Netherlands). An external heater cooler unit (Ecoline RE104, Lauda, Germany) was connected to the water reservoir. To test if fluid could flow out of the bag where the liver would be placed in, red food coloring was added to the perfusate. After the color leak test, a porcine liver was put into the PE bag, and the PV was cannulated and connected to the small perfusion circuit. The return line was placed at the bottom of the inner bag. It was tested if it was possible to increase the temperature of the liver by increasing the temperature of the water reservoir and keep the temperature stable.

2.4 Connecting the prototypes to the Liver Assist

The disposable of the Liver Assist (XVIVO abdominal, the Netherlands) was adapted to fit the prototypes (Figure 8). The bowl of the Liver Assist disposable was removed from the perfusion circuit and extra tubing (1/4-inch) was added to bridge the added distance in the perfusion circuit. An additional return line for the IVC to the perfusate reservoir (XVIVO abdominal, the Netherlands) was added. A patient monitor (SC9000XL, Siemens, Germany) was connected to the IVC line to monitor IVC pressure via a pressure transducer (Merit Medical Systems, Inc., USA). A return line for leaked perfusate was created from two PV cannulas (25 Fr, XVIVO, the Netherlands), which were connected to a Y-connector and tubing, and the cannulas were placed at the bottom of the PE bag.

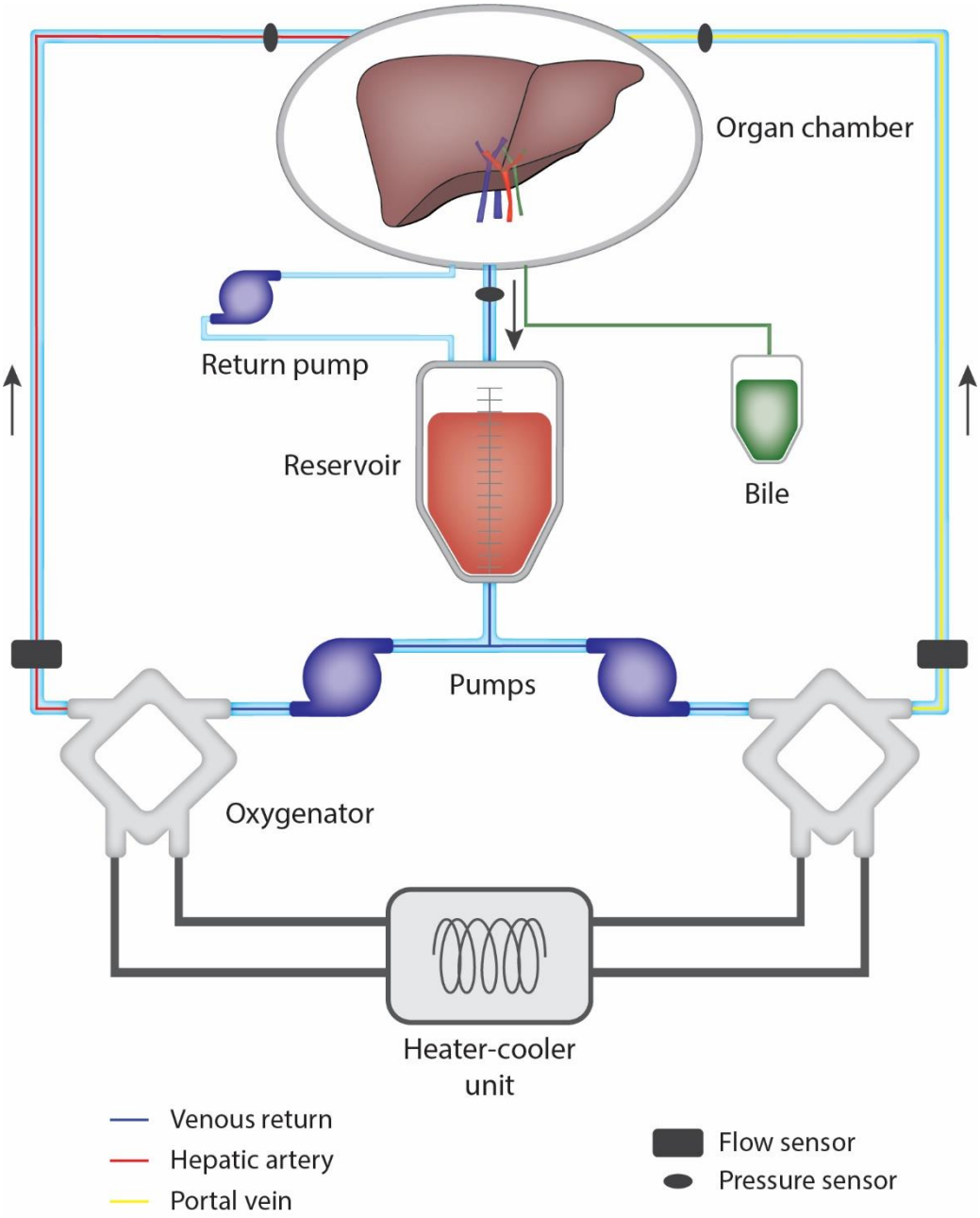


Figure 8. *A schematic overview of the perfusion circuit. The organ chamber is substituted for either prototype 1 or prototype 2.*

2.5 Porcine liver perfusion with varying water levels

The PV, HA and IVC of a porcine liver were connected to the perfusion circuit filled with NaCl 0,9% (Baxter, USA) and food coloring. The plastic container surrounding prototype 2 was filled with 19 centimeters of water, the maximum amount reaching up to the rim of the container, partially submerging the liver. Once the perfusion was stable, meaning stable flows on both the PV and HA with the desired pressure set, the water from the container was taken out until the liver was laying down instead of being submerged. After stabilizing the flows again, water was added to the container centimeter by centimeter, until the 19 centimeter was reached again. Pressures and flows of the HA, PV and IVC were monitored and recorded, as was the fluid level of the reservoir.

2.6 Human liver perfusion

COR-NMP was performed with both prototypes following the clinical DHOPE-COR-NMP protocol adapted from Van Leeuwen *et al.*[38,46]. In short, an hour of COR was followed by 2.5 hours of NMP, DHOPE was not performed. Table 2 provides information on the perfusate composition and perfusion settings during these two phases. Flow and pressure data of the human liver perfusions was recorded by the Liver Assist.

Table 2. *Perfusate composition and perfusion settings during COR-NMP.*

	COR phase	**NMP phase**
Perfusate composition	3 units of red blood cells (Sanquin, the Netherlands) 500 mL gelofusine (B. Braun, Germany) 250 mL albumin 20% (Sanquin, the Netherlands) Supliven (Fresenius Kabi, Germany) Metronidazole (Baxter, USA) Cefazolin (Mylan, USA) Cernevit (Baxter, USA) Glutatione (Biomedica Foscama, Italy) Clinimix N14G30E (Baxter, USA)	
Pressure set	PV: 3-11 mmHg HA: 20-60 mmHg	PV: 6-11 mmHg HA: 35-60 mmHg
Aimed flows	PV: 20% - 100% of liver weight HA: 80-400 ml/min	PV: 90-100 % of liver weight HA: 300-500 ml/min
Temperature	20 °C – 37 °C	37 °C
Duration	1 hour	Decision moment at 2.5 hours

2.6.1 Human liver perfusion with varying water levels in prototype 2

The perfusion was performed as described in section 2.5, with the maximum amount of water reaching the rim of the plastic container being 23 centimeters, partially submerging the liver. After 1 hour and 10 minutes of NMP, the experiment with varying water levels was performed. The starting level of water was 6 centimeters, and the container was filled centimeter by centimeter until the water level reached 23 centimeters again.

2.6.2 Liver maximum capacity test

The liver maximum capacity (LiMAx) test was performed on the first human liver following the method of Schurink *et al*[47]. ^{13}C- methacetin (Landesapotheke Salzburg, Germany) was injected after 1 hour of NMP. The dosage of ^{13}C- methacetin was calculated with the following formula:

$$^{13}\mathrm{C} - \text{methacetin dosage} = \mathrm{LW}^{\frac{4}{3}} * 2.189 * 10^{-3} \qquad \text{(Formula 1)}$$

The change in the $^{13}CO_2$:$^{12}CO_2$ ratio was recorded using the ^{13}CORLab (ArgosMED GmbH, Germany) and displayed as delta over baseline (DoB). Additional information on calculating the LiMAx values is provided in the supplementary information.

2.6.3 Indocyanine green clearance test

The ICG plasma disappearance rate (PDR) during NMP (NMP-PDR) as described by Schurink *et al.* (manuscript in preparation), is a hepatocyte function test. The indocyanine green (ICG) clearance test was performed in both human livers following the method of Schurink *et al.* (manuscript in preparation). In short: 20 mg of ICG dye (Diagnostic green GMbH, Germany) was dissolved in 4 ml of sterile water and was administered into the PV after 1 hour of NMP. Samples from the PV were taken at 2, 2.5, 3, 4, 5, 6, 7, 10, and 15 minutes after administration in EDTA blood collection tubes. The blood samples were sealed off from light and stored on ice until processing. Blood collection tubes were placed in a centrifuge (1500 G, 15 minutes, RT). ICG was measured in the supernatant by measuring the optical absorbance at 805 nm. 3·100 µL was pipetted in a 96-well plate (ThermoFisher, USA) and absorbance was measured using an Infinite pro 200M plate reader (Infinite M Nano; Tecan Trading AG, Switzerland).

2.6.4 Sampling

Wedge biopsies were taken before and after perfusion. The biopsies were fixed in 4% formaldehyde (Fresenius Kabi, Germany), before they were embedded in paraffin. Blood samples were taken every half hour during all human liver perfusions. The samples were collected in EDTA, citrate and SST blood collection tubes (BD, USA). After collection, the blood collection tubes were placed in a centrifuge and the serum/plasma was stored at -80°C until processed.

2.6.5 Histology

Paraffin embedded biopsies were sectioned at 4 µm using a microtome (Leica, Germany). Hematoxylin-eosin (HE) staining was performed by the pathology department following standard procedures. For immunohistochemistry, the sections were first deparaffinized and rehydrated by using xylene and ethanol (2·100%,1·96%, 1·70%) series. Heat-induced epitope retrieval was performed using TRIS-EDTA buffer (pH=9.0). Slides were incubated in normal serum for 1 hour before overnight incubation with primary antibodies at 4°C. An overview of the immunochemistry is provided in Table 3.

Table 3. *Immunological staining.*

Target	Reactivity	Raised in	Dilution	Brand
Albumin	Anti-Human	Mouse	1:400	Sigma
Caspase 3	Anti-Human	Performed by pathology	Performed by pathology	Performed by pathology
CD-31	Anti-Human	Performed by pathology	Performed by pathology	Performed by pathology
Cytokeratin-7	Anti-Human	Mouse	1:200	Dako

Albumin staining is positive for hepatocytes, CD-31 is positive for endothelium, cytokeratin-7 is positive for cholangiocytes and caspase 3 is positive for apoptosis, but other types of immediate cell death could also be present, such as necrosis and necroptosis[48].

Slides were washed using PBS before incubation (1 hour, RT) with HRP-conjugated ready-to-use secondary antibodies (Agilent, USA). DAB substrate was incubated for 2 minutes. All slides were counterstained with hematoxylin, before sections were dehydrated in a reversed ethanol series and xylene. Pertex was used to mount a slide cover. CD-31 and Caspase-3 staining were performed by the pathology department.

2.6.6 Serum analysis

From both human livers, blood samples were analyzed at the beginning of NMP with the prototype, and at the end. Analysis of the serum was performed by the Erasmus MC Triallab. Serum from SST blood collection tubes was analyzed for aspartate aminotransferase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (GGT).

3. Results

3.1 Donor characteristics

For both human research livers, the donor characteristics are provided in Table 4.

Table 4. *Donor characteristics.*

	Age	Sex	BMI	DCD/ DBD	fWIT (minutes)	WIT (minutes)	CIT (hours)	Reason for graft decline
Liver 1	54	Male	28	DBD	-	-	5,25	Failed NMP criteria
Liver 2	69	Female	27	DCD	25	31	25,3	Multiple cysts

The first liver was from a 54-year-old male DBD donor and weighed 3100 grams at the end of SCS. A photo of the liver is provided in Figure 9A. The liver was declined for transplantation because it did not meet the NMP criteria described by Van Leeuwen et al.[38]. In short, the criteria that need to be met after 2.5 hours of NMP are: arterial lactate <1.7 mmol/l, arterial pH between 7.35 and 7.45, bile production >10 ml and a bile pH >7.45. In addition, the difference between the bile and perfusate is also taken into account: a difference of at least 0.1 in pH, and a minimum difference of 5 mmol/l for glucose and bicarbonate need to be reached. Liver 1 did not sufficiently clear lactate and was therefore declined for transplantation. Liver 2 was from a 69-year-old female DCD donor and the liver was declined for transplantation because there were multiple cysts visible on a CT scan. A photo of this liver is provided in Figure 9B. The CIT of this liver was prolonged following the protocol of another DHOPE-COR-NMP experiment that was performed before the experiment of this master thesis. The liver weighed 1600 grams at the start of perfusion.

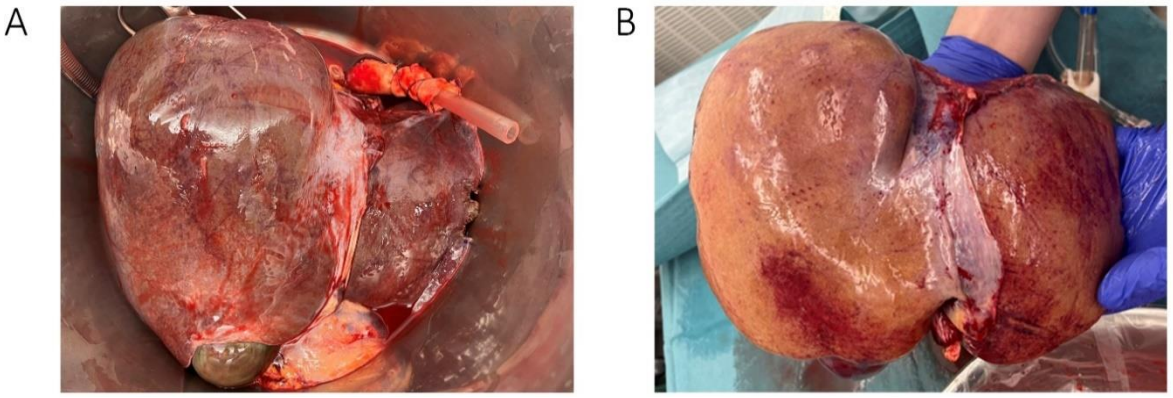


Figure 9. *Human research liver 1 (A) and human research liver 2 (B) after perfusion 1.*

In Table 5 an overview of the two livers and the performed perfusion experiments is given. From now on, the perfusions performed with the dedicated clinical disposables will be referred to as perfusion 1, and the perfusions performed with the prototypes as perfusion 2.

Table 5. *An overview of the human livers and the performed perfusions.*

	Perfusion 1	Perfusion 2
Liver 1	Research DHOPE-COR-NMP on the Liver Assist with the dedicated clinical disposable	COR-NMP with prototype 1
Liver 2	Clinical NMP on the OrganOx with the dedicated clinical disposable	COR-NMP with prototype 2

3.2 Temperature control and leak test

Submerging a liver in water could not only reduce gravitation pressure necrosis but could also allow for more precise control of the temperature of the liver. To test this hypothesis, a porcine liver was placed in a PE bag and connected to a simple perfusion circuit through the PV only. Both water and perfusate were at room temperature (20.4 °C) before the start of the experiment. After 1 hour of perfusion, the temperature of the water reservoir stabilized at 38.4°C and the surface of the liver was measured at 36.2°C. The surface of the liver did not reach the same temperature as the surrounding water reservoir, which could be due to the fact that the perfusate itself was not heated by a heat exchanger. However, this normothermic temperature range is sufficient to monitor metabolic activity of the liver and, therefore, allows for functional testing of the liver.

3.3 Prototype 1

Prototype 1 consisted of an inner bag for the liver to be placed in, the liver reservoir, and an outer bag to function as a water reservoir (Figure 10A). Five tubing connectors were added for connecting the PV, HA, IVC, return and pressure lines Figure 10B). The prototype fulfilled all requirements mentioned in Supplementary Table 1.

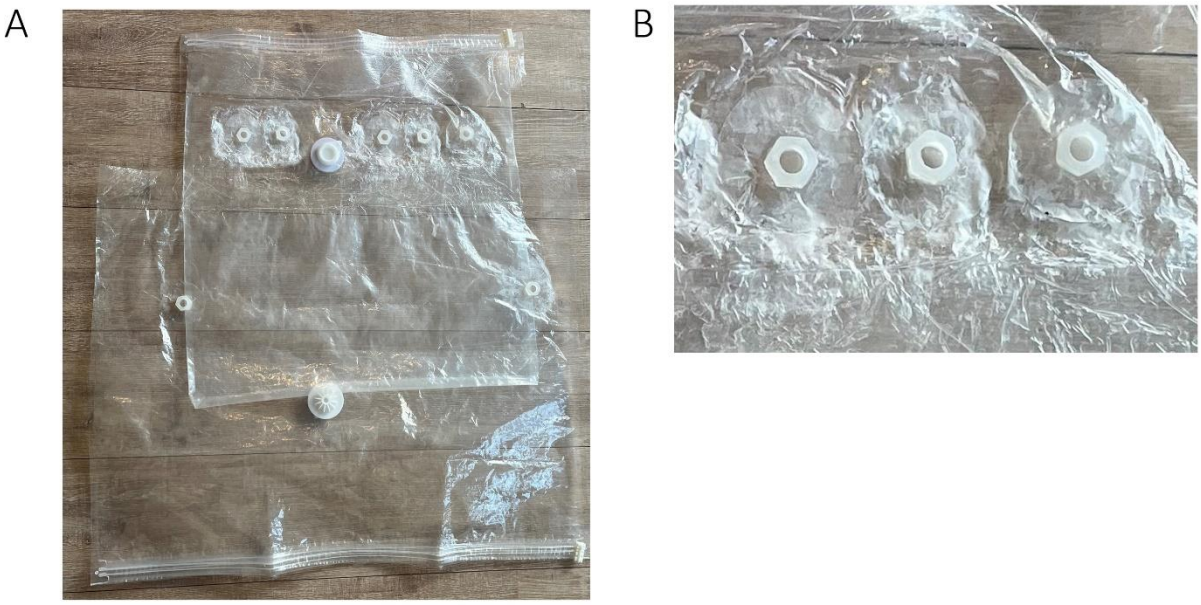


Figure 10. *Prototype 1 (A) and the tubing connectors (B).*

The prototype was placed in a transparent 40 L polypropylene household storage box, to keep it on top of the Liver Assist and prevent it from rolling off. There were no leaks in the prototype, but during the temperature and leak test with prototype 1, it was observed that the liver and the inner bag were pushed towards the surface, not submerging in the water. To solve this issue, two points of the inner bag were melted into the bottom of the outer bag. As a result, points of the inner bag were pulled down (Figure 11A). In addition, it was observed that the return line from the water reservoir back to the thermo unit was prone to air locks, interrupting heat exchange. Therefore, a long venous cannula was attached to the tube connector inside the outer bag to ensure no airlock was created in the line returning to the thermo unit (Figure 11B). When using this prototype, all vessels were cannulated, and it was a closed loop perfusion system.

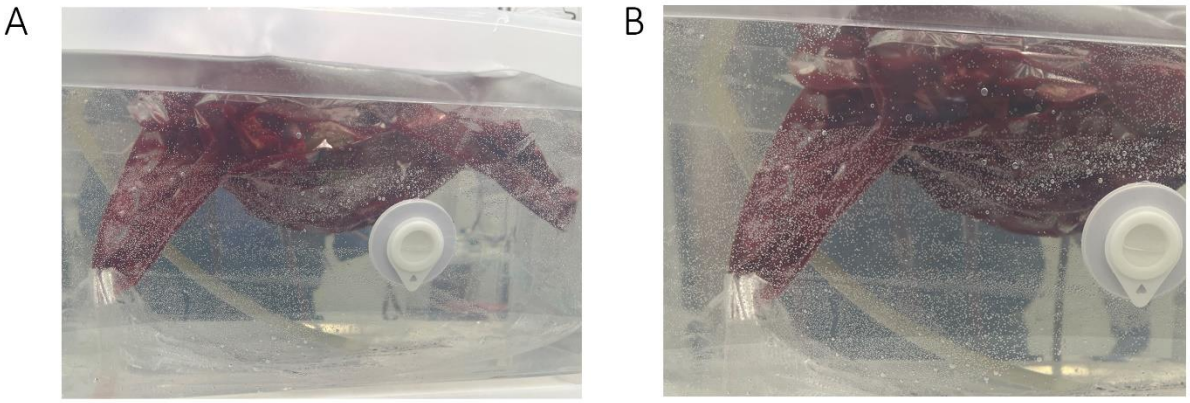


Figure 11. *The corner of the inner bag was melted into the outer bag, keeping the inner bag under water (A). The venous cannula added into the outer bag to prevent an airlock (B).*

3.3.1 Human liver perfusion

COR-NMP was performed using prototype 1 with a human research liver. The outer bag was continuously filled with the same amount of water that was warmed by the thermo unit. During COR, the pressures on both the PV and HA were gradually increased from 2 to 11 mmHg and from 20 to 44 mmHg, respectively. The corresponding flow change of the PV was from 336 ml/min to 700 ml/min. At the beginning of the COR, it was noticed that multiple side branches of the artery were leaking, which resulted in flows of more than 1000 mL/min. This can be seen at the beginning of perfusion in Figure 12A. This leak was controlled after 5 minutes of perfusion and the flow returned to 80 ml/min. After 1 hour of COR, the flow of the HA had increased to 200 ml/min. During NMP, the pressure of the PV rose only 1 mmHg to 12 mmHg and the flow increased from 700 ml/min to 860 ml/min. The HA pressure set was increased from 44 mmHg at the end of the COR phase, to 65 mmHg at 170 minutes of perfusion, after which it was kept at 65 mmHg until the end of perfusion. The flow of the HA increased from 200 ml/min to 300 ml/min during NMP. The flows and pressures of this perfusion can be found in Figure 12. The combination of warming the perfusate with the Liver Assist, and warming the surrounding water with the thermo unit, led to a liver surface temperature of 37.1 °C after 60 minutes of perfusion.

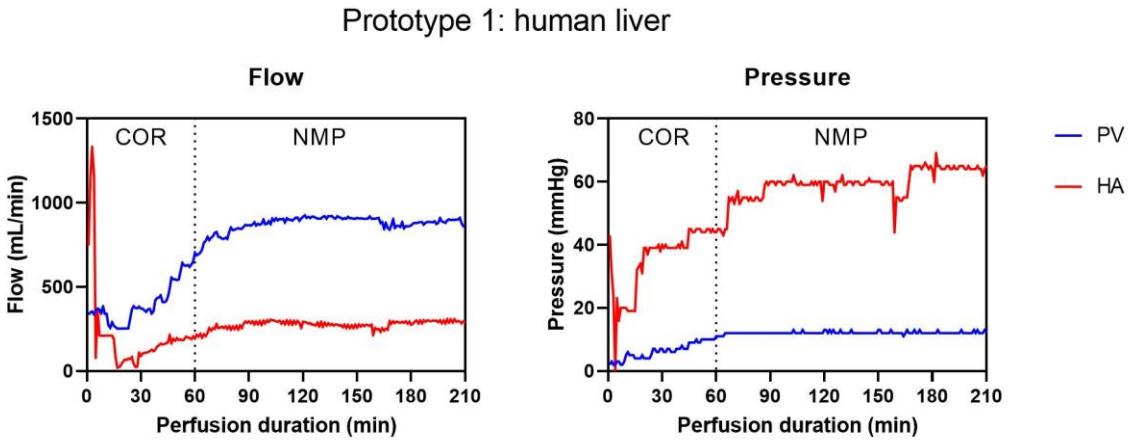


Figure 12. *Flow and pressure data of the PV and HA during human perfusion with prototype 1.*

3.4 Prototype 2

Prototype 2 consisted of a singular PE bag, which acted as the liver reservoir (Figure 13A). The bottom of this prototype was clamped between two wooden slats, to which weights could be added to keep the liver (partially) submerged. Just as in prototype 1, five tubing connectors were added underneath the zip lock (Figure 13B). The prototype was free from any leaks and placed in a transparent storage container, which functions as the water reservoir surrounding the liver. All requirements in Supplementary Table 1 were met. This prototype was part of a closed loop perfusion circuit, and all vessels were cannulated.

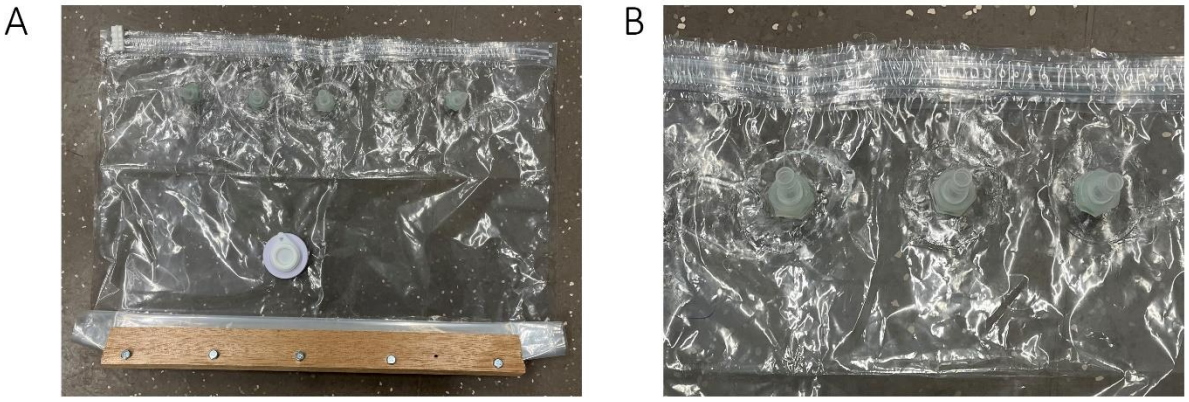


Figure 13. *Prototype 2 (A) and the tubing connectors (B).*

3.4.1 Porcine liver perfusion with varying water levels

In order to test the second prototype and the effect of different water levels on the perfusion, a porcine liver was placed inside the PE bag and connected to the Liver Assist. This machine perfuses organs based on certain (preset) pressures and tries to maintain those target pressures. Perfusion was performed for 10 minutes with 19 centimeter of water in the container. At the end of these 10 minutes of perfusion, the PV flow was 300 ml/min at a pressure of 4 mmHg, the flow on the HA artery after 10 minutes was 200 ml/min with a pressure of 29 mmHg (Figure 14). The IVC pressure was 0 mmHg.

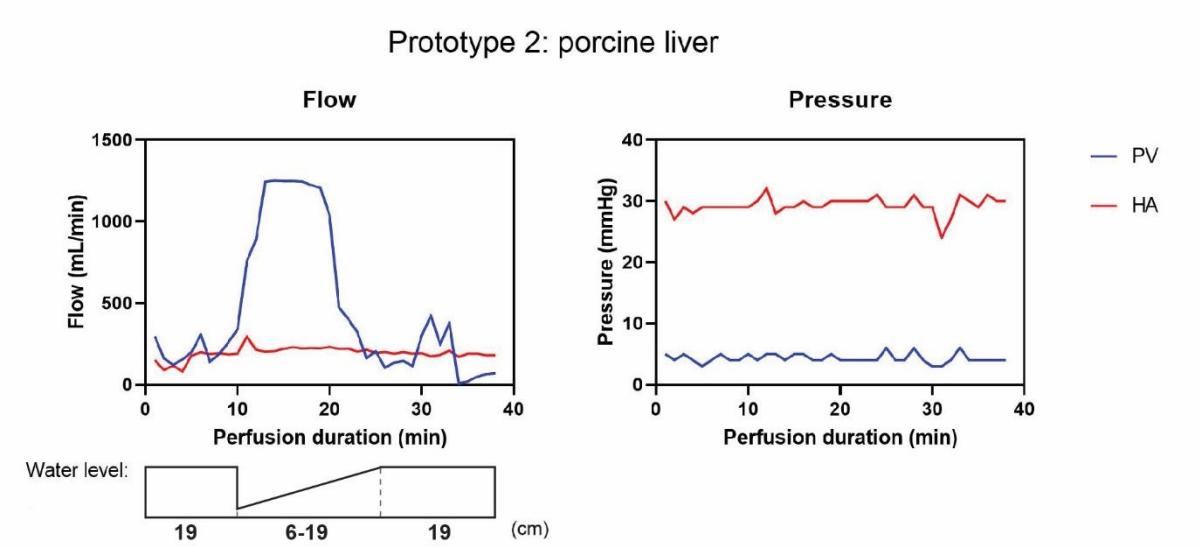


Figure 14. *Flow and pressure data of the porcine liver perfusion with prototype 2.*

After these 10 minutes of perfusion, water was taken out of the container, until the liver was laying down on the bottom, which was at 6 centimeters of water (Figure 15A). Water was added centimeter by centimeter until the water level reached the rim of the container, which was at 19 centimeters (Figure 15B).

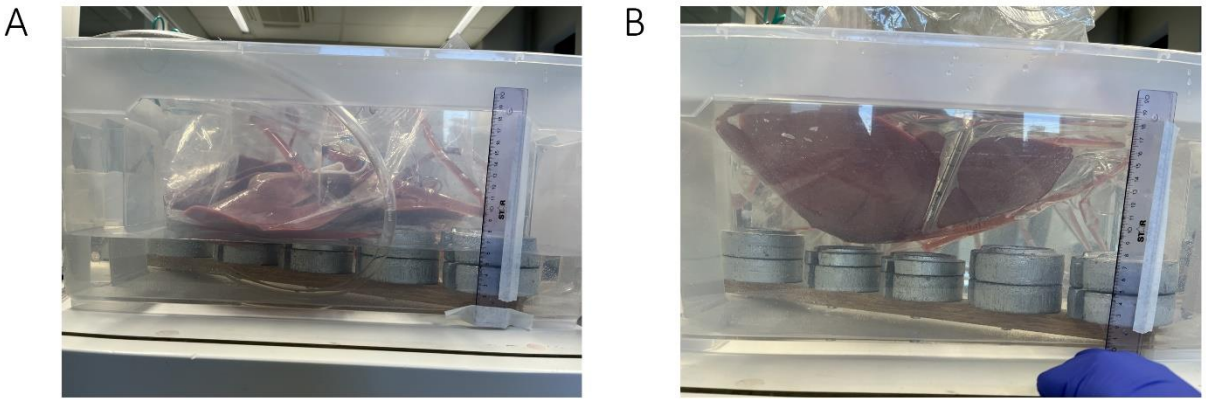


Figure 15. *The container with 6 centimeters of water, with the liver laying down on the bottom(A), and the container with 19 centimeters of water, lifting the liver of the bottom (B).*

With only 6 centimeters of water in the container, the PV flow increased up to 1250 ml/min and the PV pressure fluctuated between 4 and 5 mmHg (Figure 14 and Figure ***16***). The flow of the PV dropped significantly to 430 ml/min at 13 centimeters of water, after which the flow slowly decreased until it stabilized again at 180 ml/min with 19 centimeters of water in the container (Figure 16). The IVC pressure at 6 cm of water was 0 mmHg and remained at 0 mmHg until 12 centimeters of water was reached. From 12 centimeters to 19 centimeters the IVC pressure decreased to -6 mmHg (Figure 16). The HA flow was not influenced by the changes in water levels and remained stable around 220 ml/min, as can be seen in Figure 16.

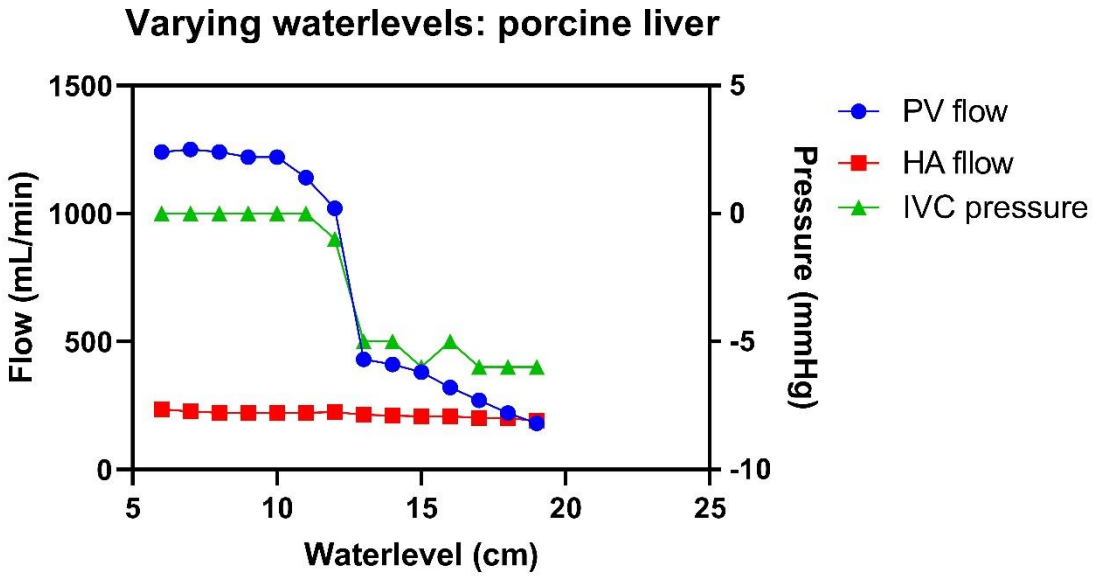


Figure 16. *The influence of varying water levels on the PV flow, the HA flow and the IVC pressure.*

3.4.2 Human liver perfusion with varying water levels

COR-NMP was performed with a human research liver with 23 centimeters of water in the container. During the COR phase of one hour, the PV pressure was gradually increased from 5 to 12 mmHg, leading to an increase in flow from 212 to 760 ml/min at 60 minutes. The pressure on the HA was increased from 30 to 65 mmHg within the first hour, with an increase in flow from 7 to 308 ml/min at 60 minutes. Flow and pressure data of the perfusion is provided in Figure 17.

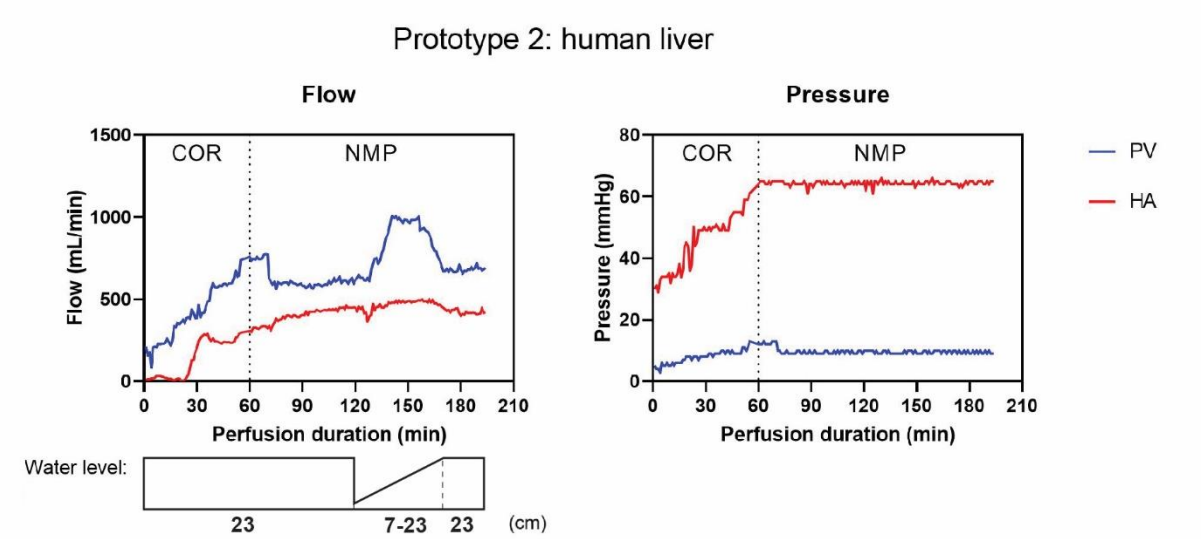


Figure 17. *Flow and pressure data of the human research liver perfusion with prototype 2.*

After 70 minutes of perfusion, the PV pressure setting was changed from 12 to 10 mmHg, leading to a decrease in flow. This effect can be seen in Figure 17 just after the dashed line at 60 minutes of perfusion. After this change in setting, the pressures were not changed and after 120 minutes of perfusion, the PV flow stabilized around 600 ml/min and the HA flow increased to 450 ml/min (Figure 17). The IVC pressure after 120 minutes of perfusion was -3 mmHg.

At 120 minutes of perfusion the changing water level experiment was started. Water was taken out of the reservoir until 7 centimeters of water was left and the liver was laying at the bottom of the container (Figure 18A). The container was filled centimeter by centimeter, until the 23 centimeters was reached again (Figure 18B).

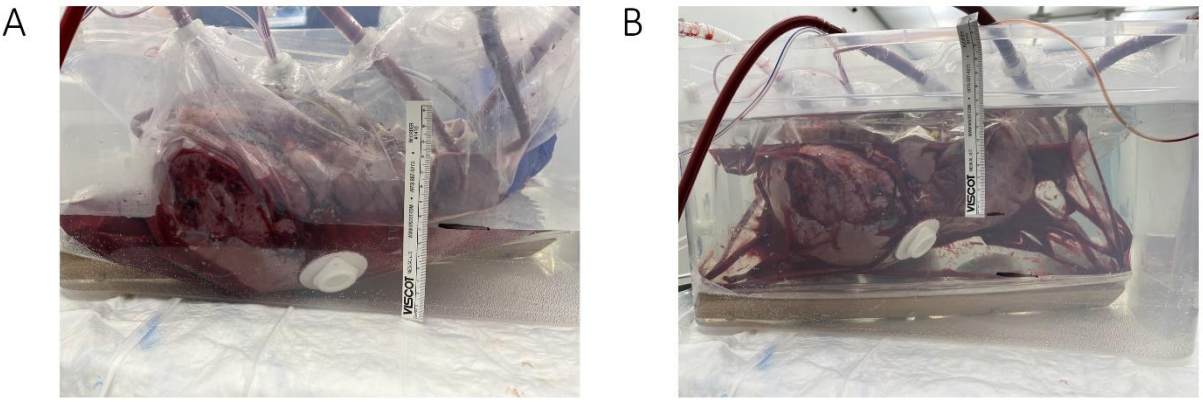


Figure 18. *The container with 7 centimeters of water, with the liver laying down on the bottom(A), and the container with 23 centimeters of water, lifting the liver of the bottom (B).*

With 7 centimeters of water, the PV flow increased to 940 ml/min and the HA flow increased only slightly to 480 ml/min (Figure 17). Additionally, without the pressure from the water reservoir acting on the liver, more leakage from the cannulation and biopsy sites was observed and the perfusate level in the reservoir dropped. After stabilization, water was added to the container centimeter by centimeter. From 7 to 12 centimeters of water, the flows increased slightly: the PV flow increased to 980 ml/min and the HA flow increased to 500 ml/min (Figure 19). The pressure in the IVC slowly decreased from 2 to 0 mmHg. At 12 centimeters of water, indicated by the dashed line in Figure 19, the perfusate level in the reservoir had dropped drastically, and the flow of the return pump was increased. The effect of the return pump on the IVC pressure can be seen; at 12 centimeters of water, the IVC pressure had dropped to 0 mmHg. After increasing the flow of the return line, the IVC pressure rose to 2 mmHg, and it started to decrease again with 18 centimeters of water in the container. When the water level reached the rim of the container again, which was 23 centimeters of water, the IVC pressure reached -3 mmHg. At 12 centimeters of water, the PV flow also started to decrease and dropped to 660 ml/min with 23 centimeters of water. The HA flow remained between 440 and 500 ml/min and was not as strongly influenced by the changing water levels in the container as the PV flow. With more water in the container, above 16 centimeters, less leaking was observed, and it was observed that the perfusate level in the reservoir rose.

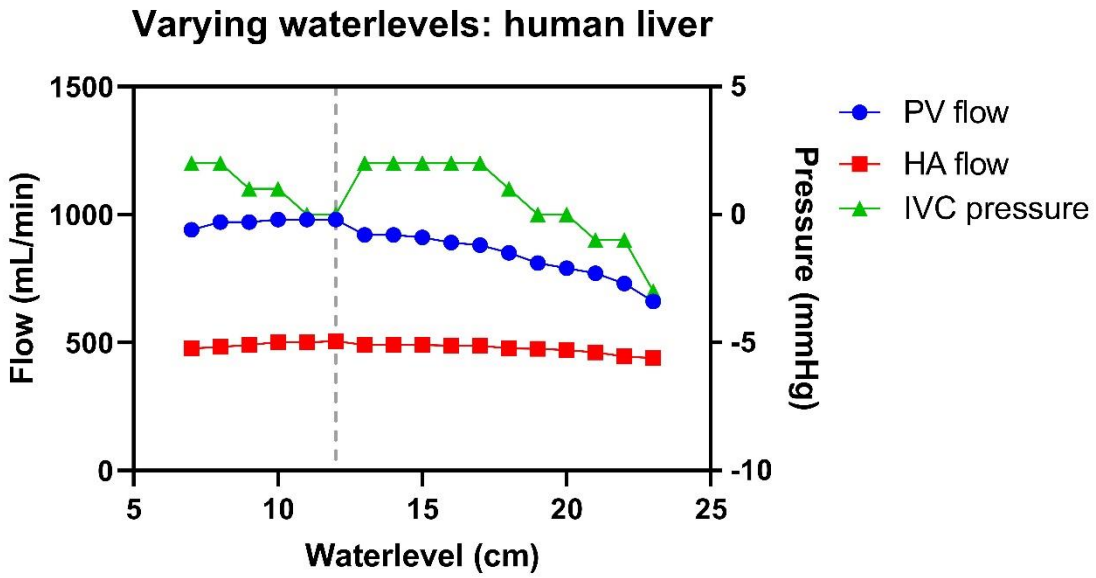


Figure 19. *The influence of varying water levels on the PV flow, the HA flow and the IVC pressure.*

3.5 Liver maximum capacity test

The LiMAx test was performed twice on the first human research liver with prototype 1. The first test was performed during perfusion 1 on the Liver Assist with the original disposable following the clinical protocol of Van Leeuwen *et al.* and had a LiMAx value of 260 µg/kg/h[38]. This first test was considered as base line for this liver. The second test was performed during perfusion 2 using prototype 2 and had a value of 369 µg/kg/h. A higher LiMAx score is correlated with a better metabolic function, as more substrate is metabolized by the liver. It is important to note that during perfusion 1, the liver is pressed down into the net of the Liver Assist due to gravity, whereas in prototype 1, the liver is submerged or even floating. The improved LiMAx score could be due to improved perfusion of the peripheral regions of the liver. However, these differences between the Liver Assist organ chamber and prototype 1, need to be assessed further to test this hypothesis.

3.6 Indocyanine green clearance test

During perfusion of the first human research liver, the NMP-PDR after 1 hour of NMP was 12.5 %/l·kg during perfusion 1 and 22.3 %/l·kg during perfusion 2 with prototype 1. The first human research liver shows an increase in NMP-PDR when using prototype 1, when the liver is partially submerged. This correlates with the improved LiMAx score in prototype 1 compared to the Liver Assist organ chamber. The improved NMP-PDR and LiMAx score show that the liver did not deteriorate during perfusion with prototype 1. In prototype 2, with liver 2, the NMP-PDR was 10.5 %/l·kg after 1 hour of NMP during perfusion 1 with the dedicated OrganOx organ chamber. During perfusion 2, with prototype 2, the NMP-PDR was 6.2 %/l·kg after 1 hour of NMP. Schurink *et al.* presented a NMP-PDR cut off value of 13.3 %/l·kg (manuscript in preparation). Higher scores are indicative for absence of post-transplant hepatocellular or biliary failure in ECD livers. The second human liver was declined for transplantation based on failed lactate clearance during NMP. The NMP-PDR of the second liver during perfusion 1 was 10.5 %/l·kg and supports the choice to not transplant this liver. The ICG absorption graphs for all four perfusions can be found in the supplementary information.

3.7 Blood samples

Levels of liver injury markers AST, ALT and GGT of both human livers are shown in Figure 20. In liver 1, using prototype 1, ALT levels started at 2837 and increased up to 3558 U/l after 150 minutes of NMP. AST measurements in the samples of the first liver were not possible due to a hemolytic sample, indicating a high level of damaged red blood cells. The initial GGT levels at the start of NMP were also higher in the first liver, starting at 42 and at the end of NMP reaching 113 U/l. In liver 2, using prototype 2, the starting levels of ALT were lower compared to liver 2, ALT increased from 774 to 821 U/l over 150 minutes of NMP. AST increased from 1065 to 1465 U/l. GGT showed an increase of 15 to 55 U/l during NMP. These results indicate higher injury in liver 1, compared to liver 2, which could be explained by the long CIT of almost 24 hours in the first liver. Even though the starting levels in liver 1 are higher, the increase in levels of injury markers is almost the same for both livers (Figure 20).

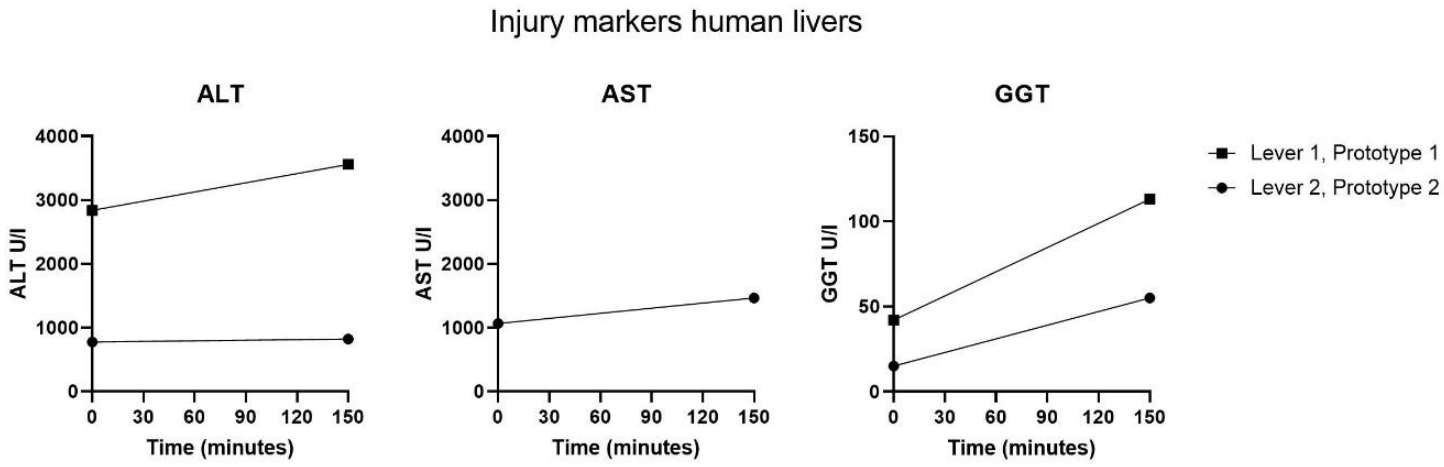


Figure 20. *Injury markers for both human livers after perfusion with either prototype 1 or 2.*

3.8 Liver biopsies

Figure 21 shows representable HE slides of the second human research liver at three different time points. The donor had a BMI of 27, and the liver weighed 1600 grams. No fat droplets were seen (Figure 21A, B and C). The sinusoids are more pronounced in liver 1 than in liver 2 and the sinusoids became more pronounced after the perfusion experiments, which could indicate that the hepatocytes have shrunk, or perfusion pressures were too high (Figure 21C). The second human research liver was from a donor with a BMI of 28 and the liver weighed more than 3000 grams. Macroscopically the liver had a yellowish color at the end of the CIT. In the biopsy at the end of SCS, fat droplets can be seen (Figure 21D) and this pattern can be seen throughout the rest of the biopsies (Figure 21E and F). At the end of perfusion 2, the liver was not flushed, and red blood cells can still be seen in the sinusoids, which can give a distorted picture of the size of the sinusoids (Figure 21F). After perfusion 2, necrotic areas were seen, of which a representable slide is added in Supplementary figure 3. These areas could explain the increase in liver injury markers shown in Figure 20.

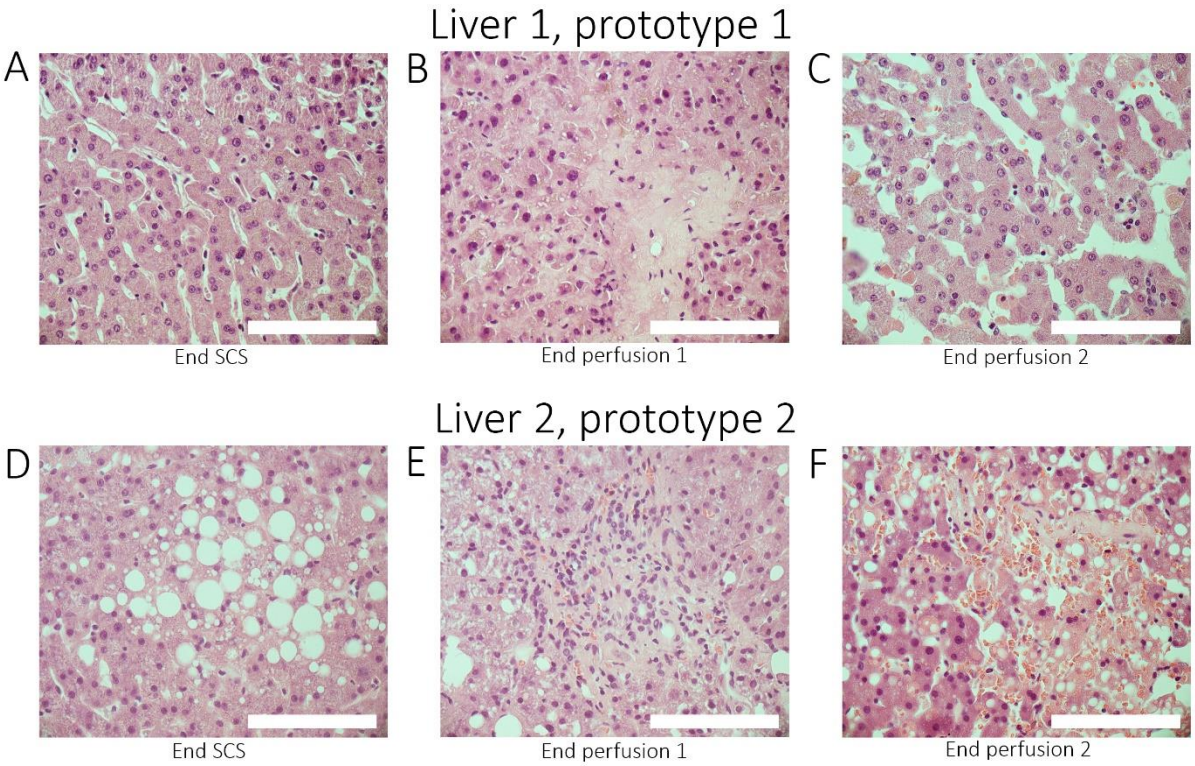


Figure 21. *Representative HE slides from both human livers. A and D are the end of SCS, B and E after the first perfusion experiment and C and F after the second perfusion experiment. All scale bars represent 100 µm.*

After the second perfusion the hepatocytes stained positive for albumin, the endothelial cells stained positive for CD31 and the cholangiocytes stained positive for CK7 (Figure 23). When looking at the albumin staining, a difference can be seen between liver 1 and 2 (Figure 23A and D). More homogenous staining can be seen in liver 1 compared to liver 2. In liver 2 less positive hepatocytes were visible compared to liver 1. No major differences were seen when looking at the endothelial, apart from the difference in sinusoidal size (Figure 23B and E). The cholangiocytes were seen in both livers after two perfusions (Figure 23C and F). This indicates that all three cell types were still present in both livers after 2 perfusions. However, it is unknown if the cells are still functional.

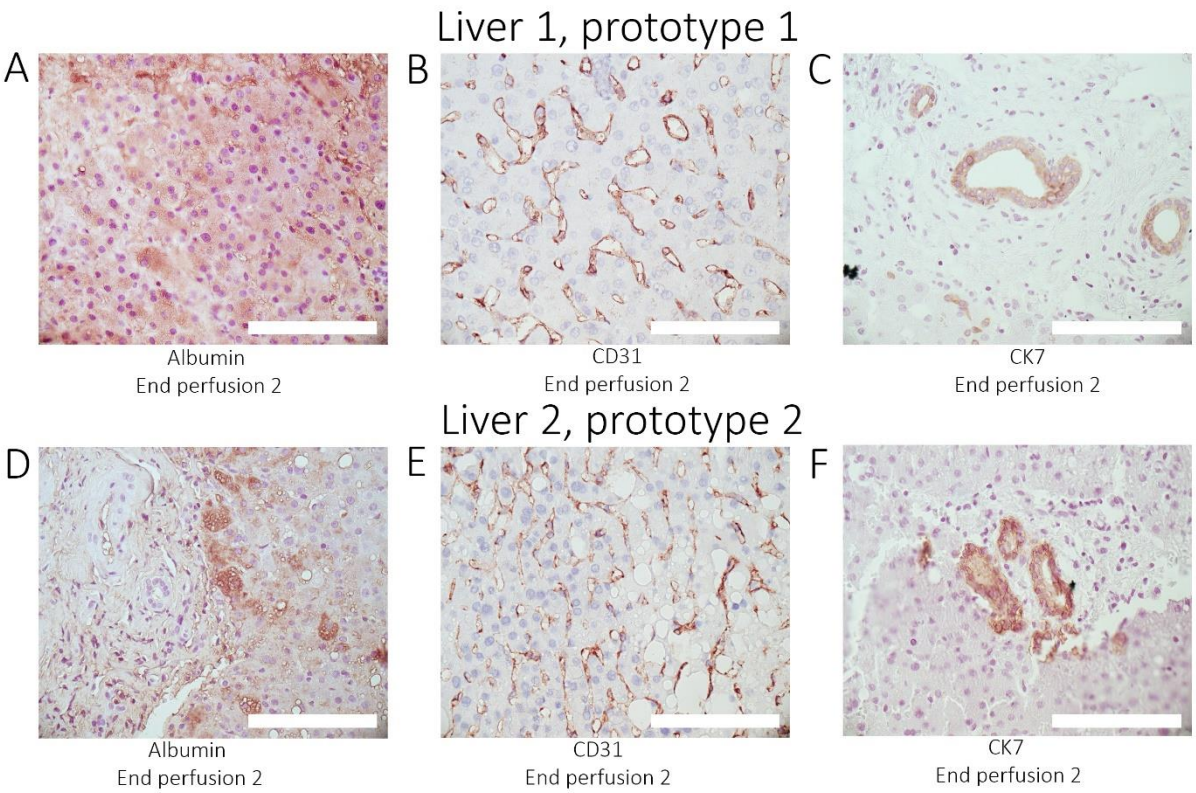


Figure 23. *Representative slides from both human livers after two perfusions. A and D show albumin staining, B and E show CD31 staining, C and F show CK7 staining. All scale bars represent 100 µm.*

Caspase 3 positive cells are stained pink, and an arrow was added to every slide to indicate a positive cell. Not all positive cells on a slide were indicated by an arrow. Caspase 3 positive cells can already be seen in the biopsies of both livers taken at the end of SCS (Figure 22A and D). In the representative slides, it appears that there are not more caspase 3 positive cells in the biopsies taken after the first and second perfusion (Figure 22). However, this was not quantified.

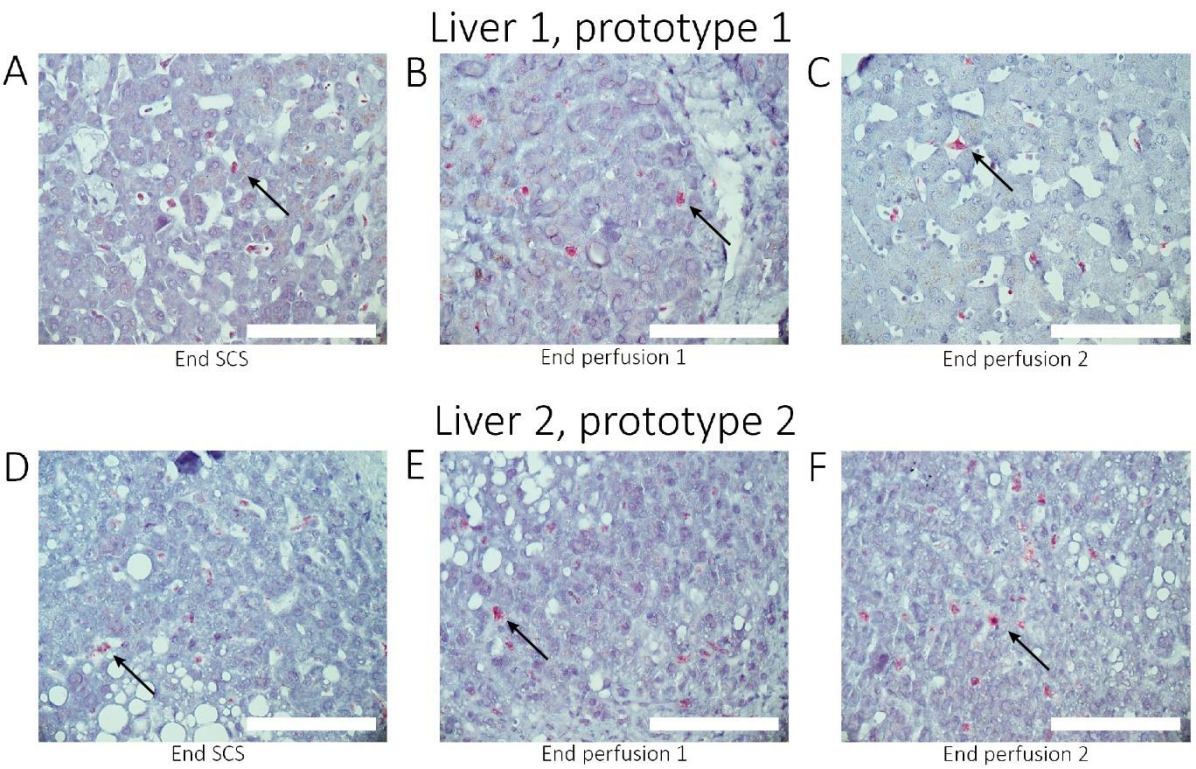


Figure 22. *Representative caspase 3 slides from both human livers. An arrow indicates a caspase 3 positive cell, not all positive cells are indicated. A and D are the end of SCS, B and E after the first perfusion experiment and C and F after the second perfusion.*

4. Discussion

In this thesis we demonstrate a new approach to the organ chamber design, which, together with other advances is required to enable long term *ex situ* liver machine perfusion. At this moment, the perfusion duration is limited by the occurrence of gravity induced pressure injuries on the liver. In an effort to prevent the occurrence of pressure injuries, two organ chamber prototypes were developed. We made the fundamental choice to base both prototypes on submerging the liver in water, and thus to let it float instead of statically laying down on a hard surface.

During testing with both prototypes, it was observed that the liver was truly floating. When the liver is afloat, the water pushes back against the parts of the liver that are in contact with water. In other words, the water can exert more equal forces on the liver. Nevertheless, letting the liver float could be sufficient to prevent pressure injuries on the liver, but it will not influence the peripheral perfusion. The choice was made to fixate both of the liver reservoirs to the bottom of the water reservoir, to be able to partially submerge the liver and to apply an outside pressure on the liver with the surrounding water. The water can exert more equal forces on the liver, and this can be extended to all sides if the liver is submerged. Potentially, this could result in a more homogeneous spread of the pressure exerted on the liver. By varying the amount of water surrounding the liver, the sponge like effect of the diaphragm could be mimicked and this could possibly influence the peripheral perfusion. In prototype 1 it turned out that it was not easy to change and control the water level. Because this prototype consisted of a bag in a bag construction, it was impossible to monitor the water level and to add a specific amount of water. Therefore, the experiment with varying water levels in the reservoir was only performed with prototype 2. During these experiments with varying water levels, it was observed that lower water levels in the water reservoir, led to higher flows in the PV and when increasing the water level, the PV flows decreased. This suggest that with lower water levels, there is less pressure from the water on the liver surface, there is more flow through the PV and the liver fills up with blood that could be distributed to the periphery. With higher water levels and thus more pressure on the liver surface, there is a lower PV flow, and it could be that less blood is distributed to the periphery. HA flows stayed relatively the same with different water levels. The difference in pressures on the PV, which lays between 10-12 mmHg, and pressures on the HA, above 60 mmHg, could explain the different effects of the water levels on the PV and HA flow. Higher pressures exerted on the liver will have less effect on a high HA pressure than on a low PV pressure. During the porcine experiment with varying water levels, there is a steep drop in PV flow after 13 centimeters of water. In the human perfusion this steep drop is not seen, but at a similar water level, 12 centimeters, the PV flow starts to drop gradually. It could be possible that with these water levels, the pressure from the water on the liver causes the periphery to close, or it could cause the PV to partially collapse. Interestingly, in both perfusions with prototype 2, when having the maximum amount of water in the reservoir, the IVC pressure was negative. There was a height difference between the liver and the perfusate reservoir and there was a substantial amount of tubing from the organ chamber to the perfusate reservoir. The IVC pressure was measured just after the liver and inside the organ chamber, thus, there is a large column of perfusate pulling on the IVC, creating this negative IVC pressure. This pulling force of the perfusate column was constant during the entire perfusion, as the reservoir height was not changed. The pressure in the IVC was influenced by the water level in the container. This could be explained by looking at the PV flows: with low water levels in the container, the PV flow was high, thus there was more flow through the IVC and therefore

higher pressures. With higher water levels, the PV flow decreased, leading to a decrease in ICV flow therefore a lower IVC pressure.

Both prototypes presented in this thesis consisted of a plastic bag for the liver to be placed in. The size of these plastic bags was bigger than the sizes of both the organ chamber of the OrganOx Metra and the Liver Assist, and the material of the bag is less rigid compared to the clinical organ chambers. This allows for easy repositioning of the liver, but also provides the opportunity to place the liver in another position than inverted and resting on the front side. The liver could be placed in a more physiological position, for example the position the liver would be in when a person is standing straight or laying down on their back. Changing the position of the liver over time, could prevent pressure injuries, but it could also improve the blood flow to the periphery of the liver.

The Zurich group that developed the Liver4Life used a different approach and placed the liver on a silicon mat with an inflatable balloon underneath[43]. Even though they reported no pressure injuries after 7 days of perfusion imaged with 18-fluorodeoxyglucose PET-CT, it could be argued that during perfusion, the contact area of the liver surface with the mat will always be the same area and eventually gravity induced pressure injury will occur. A possible solution could be creating multiple compartments in the inflatable balloon, which then can be inflated and deflated separately. This mimics the idea of an anti-decubitus air mattress used to prevent bedsores in patients who are bedridden. By inflating and deflating different compartments of the balloon under the liver, the contact area of the liver surface changes and there will not be only one area of contact. However, all gravitational force created by the weight of the liver is still exerted on those (limited) contact areas between the balloon and the liver. Even though multiple balloons can create a more dynamic surface for the liver, peripheral perfusion can still be limited in those contact areas on which most of the weight is resting. It would be interesting to look into this direction when further developing the organ chamber.

In this thesis we made a first attempt to assess the role of submerging the liver in a fluid, instead of letting it rest on a hard surface. With simple materials such as big zip lock PE bags and tubing connectors, a working prototype can be created. The prototypes can be closed, and the liver is not submerged in the perfusate to minimize the microbial risk. It has been established that varying the water level surrounding the liver influences the PV flow and IVC pressure and could potentially influence the peripheral perfusion. Furthermore, another benefit of submerging the liver in water is additional temperature control. Currently used clinical perfusion machines control temperature by cooling or heating the perfusate through heat exchange in the oxygenator. During the COR phase, the liver is slowly rewarmed by the perfusate, from the inside. Additionally, heating the water bath that surrounds the liver could provide a more controlled and homogenous rewarming of the whole liver. This characteristic could not only be used for rewarming the organ, but it could also be used to cool the liver at the end of perfusion or in case of machine failure cool the liver more quickly.

A limitation of this master thesis is that both human research livers were first perfused using the commercially available disposables, in which they laid statically on a flat surface. The damage that possibly occurred during these perfusions could not be undone using the developed prototypes. When looking at these injuries, it would be difficult to distinguish between injuries inflicted by the organ chambers of the machines or inflicted by the developed prototypes. Up next is performing perfusions with a dedicated human research liver that was not already subjected to another

perfusion. Another limitation was, that in this master thesis, quantification of the pressure injuries and peripheral perfusion was not performed. To enable long term liver perfusion, perfusing the whole organ without inflicting pressure injuries, is of critical essence and quantifying the perfusion of the periphery is needed to take the next step. Optical imaging techniques could be used to visualize and quantify peripheral perfusion and pressure injuries, like laser speckle contrast imaging (LSCI) and fluorescence imaging. LSCI can be used to quantify relative changes in blood flow. With this technique, flow in the center of the liver can be compared to flow in the periphery and possible shunting between the PV and IVC can be visualized[49]. In a clinical trial, Eriksson *et al.* successfully measured and analyzed the hepatic microcirculation during 10 liver resections using LSCI[50]. To visualize perfusion defects and pressure injuries inflicted on the organ, fluorescence imaging can be used. This was demonstrated by Eshmuminov *et al.*, who showed impaired circulation in pressure areas on fluorescein images[43]. Aforementioned techniques should be considered when further developing the organ chamber, as these can dictate new requirements for the organ chamber, such as integrated (optical) sensors or optical windows for imaging modalities.

This research has raised some important questions in order to improve and develop a new prototype. It can be concluded from these result that (partially) submerging the liver is an interesting concept that could prevent pressure injuries and possibly improve peripheral perfusion. The steep decrease in PV flow when varying the water level was striking, and more livers must be perfused to see if this steep drop occurs again. To gain more insight on these perfusion changes, the varying water level experiment can be performed in both directions: first the water level is decreased centimeter by centimeter, monitoring the perfusion, and then water is added again centimeter by centimeter. Moreover, other future research could include more immediate changes in water levels, to mimic the frequency of diaphragm movement when breathing (e.g., 12 inhalations per minute). This could ultimately increase and decrease the flow through the periphery of the liver and create the sponge like effect of the diaphragm. This can be achieved by limiting the inflow and outflow of water into the water reservoir. To mimic the effect of breathing in, there must be more water in the reservoir. By closing the outflow of the reservoir, but keeping the inflow open, the reservoir will be filled and the water level will rise. When opening the outflow, but not starting the inflow again, the water level will fall, mimicking the effect of breathing out. By opening and closing the inflow and outflow intermittently, the sponge like effect can be created. It must be investigated if these more immediate water level changes are easier to create using bag in a bag principle or a bag in a container principle. Important in the next steps of developing a new prototype is including imaging techniques such as laser speckle to quantify and improve the peripheral perfusion.

In conclusion: we showed that PV flow and subsequent IVC pressure is highly dependent on the pressure of the environment acting on the liver, as the flow of the HA is not. Quantification of the flow in the liver is needed to determine if external pressure on the liver leads to an increase or decrease in peripheral perfusion.

5. References

1. Burra P, Becchetti C, Germani G. NAFLD and liver transplantation: Disease burden, current management and future challenges. *JHEP Rep*. Dec 2020;2(6):100192.
2. Adam R, Karam V, Cailliez V, et al. 2018 Annual Report of the European Liver Transplant Registry (ELTR) - 50-year evolution of liver transplantation. *Transpl Int*. Dec 2018;31(12):1293-1317.
3. Durand F, Levitsky J, Cauchy F, Gilgenkrantz H, Soubrane O, Francoz C. Age and liver transplantation. *J Hepatol*. Apr 2019;70(4):745-758.
4. Eurotransplant. Accessed 13-02-2023, 2022. https://www.eurotransplant.org/
5. Stichting NT. NTS jaarverslag 2021. NTS. https://www.transplantatiestichting.nl/files/Jaarverslag/nts-cijferbijlage-jaarverslag-2021.pdf?031832443f
6. Braat AE, Blok JJ, Putter H, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. *Am J Transplant*. Oct 2012;12(10):2789-96.
7. Flores A, Asrani SK. The donor risk index: A decade of experience. *Liver Transpl*. Sep 2017;23(9):1216-1225.
8. Jochmans I, van Rosmalen M, Pirenne J, Samuel U. Adult Liver Allocation in Eurotransplant. *Transplantation*. Jul 2017;101(7):1542-1550.
9. Barshes NR, Horwitz IB, Franzini L, Vierling JM, Goss JA. Waitlist mortality decreases with increased use of extended criteria donor liver grafts at adult liver transplant centers. *Am J Transplant*. May 2007;7(5):1265-70.
10. Merion RM, Goodrich NP, Feng S. How can we define expanded criteria for liver donors? *J Hepatol*. Oct 2006;45(4):484-8.
11. McCormack L, Dutkowski P, El-Badry AM, Clavien PA. Liver transplantation using fatty livers: always feasible? *J Hepatol*. May 2011;54(5):1055-62.
12. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation*. Aug 27 2011;92(4):373-9.
13. Schlegel A, Kalisvaart M, Scalera I, et al. The UK DCD Risk Score: A new proposal to define futility in donation-after-circulatory-death liver transplantation. *J Hepatol*. Mar 2018;68(3):456-464.
14. Linares I, Hamar M, Selzner N, Selzner M. Steatosis in Liver Transplantation: Current Limitations and Future Strategies. *Transplantation*. Jan 2019;103(1):78-90.
15. Hoyer DP, Paul A, Gallinat A, et al. Donor information based prediction of early allograft dysfunction and outcome in liver transplantation. *Liver Int*. Jan 2015;35(1):156-63.
16. Foley DP, Fernandez LA, Leverson G, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. *Ann Surg*. Apr 2011;253(4):817-25.
17. Blok JJ, Detry O, Putter H, et al. Longterm results of liver transplantation from donation after circulatory death. *Liver Transpl*. Aug 2016;22(8):1107-14.
18. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. *Biochim Biophys Acta Mol Basis Dis*. Apr 2018;1864(4 Pt B):1507-1515.
19. Meurisse N, Vanden Bussche S, Jochmans I, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. *Transplant Proc*. Nov 2012;44(9):2868-73.
20. Israni AK, Zaun D, Rosendale JD, Schaffhausen C, McKinney W, Snyder JJ. OPTN/SRTR 2019 Annual Data Report: Deceased Organ Donors. *Am J Transplant*. Feb 2021;21 Suppl 2:521-558.
21. Aufhauser DD, Jr., Foley DP. Beyond Ice and the Cooler: Machine Perfusion Strategies in Liver Transplantation. *Clin Liver Dis*. Feb 2021;25(1):179-194.
22. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. Nov 20 2014;515(7527):431-435.
23. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation*. May 1992;53(5):957-78.
24. de Rougemont O, Lehmann K, Clavien PA. Preconditioning, organ preservation, and postconditioning to prevent ischemia-reperfusion injury to the liver. *Liver Transpl*. Oct 2009;15(10):1172-82.
25. van Rijn R, Schurink IJ, de Vries Y, et al. Hypothermic Machine Perfusion in Liver Transplantation - A Randomized Trial. *N Engl J Med*. Apr 15 2021;384(15):1391-1401. doi:10.1056/NEJMoa2031532
26. Xu J, Buchwald JE, Martins PN. Review of Current Machine Perfusion Therapeutics for Organ Preservation. *Transplantation*. Sep 2020;104(9):1792-1803. doi:10.1097/tp.0000000000003295
27. Rijkse E, JN IJ, Minnee RC. Machine perfusion in abdominal organ transplantation: Current use in the Netherlands. *World J Transplant*. Jan 18 2020;10(1):15-28. doi:10.5500/wjt.v10.i1.15

28. Van Raemdonck D, Neyrinck A, Rega F, Devos T, Pirenne J. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? *Curr Opin Organ Transplant*. Feb 2013;18(1):24-33. doi:10.1097/MOT.0b013e32835c494f
29. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature*. May 2018;557(7703):50-56.
30. Hefler J, Marfil-Garza BA, Dadheech N, Shapiro AMJ. Machine Perfusion of the Liver: Applications Beyond Transplantation. *Transplantation*. Sep 2020;104(9):1804-1812.
31. Minor T, von Horn C, Zlatev H, et al. Controlled oxygenated rewarming as novel end-ischemic therapy for cold stored liver grafts. A randomized controlled trial. *Clin Transl Sci*. Dec 2022;15(12):2918-2927.
32. Aferetica, PerLife. Accessed 08/12/2022, https://www.aferetica.com/en/therapeutical-solutions/perlife-tm/
33. Organ Assist, the Liver Assist. Accessed 08/12/2022, https://www.organ-assist.nl/products/
34. OrganOx, living organs for life. Accessed 08/12/2022, https://www.organox.com/
35. TransMedics, OCS Liver. Accessed 08/12/2022, https://www.transmedics.com/ocs-hcp-liver/
36. TransMedics, Organ Care System. Accessed 08/12/2022, https://www.transmedics.com/ocs-hcp/
37. Bridge to Life Europe, VitaSmart Machine Perfusion System. Accessed 08/12/2022, https://bridgetolife.eu/vitasmart/
38. van Leeuwen OB, Bodewes SB, Lantinga VA, et al. Sequential hypothermic and normothermic machine perfusion enables safe transplantation of high-risk donor livers. *Am J Transplant*. Mar 14 2022;
39. de Vries Y, Berendsen TA, Fujiyoshi M, et al. Transplantation of high-risk donor livers after resuscitation and viability assessment using a combined protocol of oxygenated hypothermic, rewarming and normothermic machine perfusion: study protocol for a prospective, single-arm study (DHOPE-COR-NMP trial). *BMJ Open*. Aug 15 2019;9(8):e028596.
40. Brüggenwirth IMA, Lantinga VA, Rayar M, et al. Prolonged dual hypothermic oxygenated machine preservation (DHOPE-PRO) in liver transplantation: study protocol for a stage 2, prospective, dual-arm, safety and feasibility clinical trial. *BMJ Open Gastroenterol*. Jan 2022;9(1)
41. Lascaris B, Thorne AM, Lisman T, Nijsten MWN, Porte RJ, de Meijer VE. Long-term normothermic machine preservation of human livers: what is needed to succeed? *Am J Physiol Gastrointest Liver Physiol*. Feb 1 2022;322(2):G183-G200.
42. Liu Q, Nassar A, Buccini L, et al. Ex situ 86-hour liver perfusion: Pushing the boundary of organ preservation. *Liver Transpl*. Apr 2018;24(4):557-561. doi:10.1002/lt.25007
43. Eshmuminov D, Becker D, Bautista Borrego L, et al. An integrated perfusion machine preserves injured human livers for 1 week. *Nat Biotechnol*. Feb 2020;38(2):189-198. doi:10.1038/s41587-019-0374-x
44. Clavien PA, Dutkowski P, Mueller M, et al. Transplantation of a human liver following 3 days of ex situ normothermic preservation. *Nat Biotechnol*. Nov 2022;40(11):1610-1616.
45. Nassar A, Liu Q, Farias K, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: results from a large animal model. *Surg Innov*. Feb 2015;22(1):61-9. doi:10.1177/1553350614528383
46. van Leeuwen OB, de Vries Y, Fujiyoshi M, et al. Transplantation of High-risk Donor Livers After Ex Situ Resuscitation and Assessment Using Combined Hypo- and Normothermic Machine Perfusion: A Prospective Clinical Trial. *Ann Surg*. Nov 2019;270(5):906-914.
47. Schurink IJ, de Haan JE, Willemse J, et al. A proof of concept study on real-time LiMAx CYP1A2 liver function assessment of donor grafts during normothermic machine perfusion. *Sci Rep*. Dec 6 2021;11(1):23444.
48. Shi S, Verstegen MMA, Mezzanotte L, de Jonge J, Löwik C, van der Laan LJW. Necroptotic Cell Death in Liver Transplantation and Underlying Diseases: Mechanisms and Clinical Perspective. *Liver Transpl*. Jul 2019;25(7):1091-1104.
49. Uz Z, Shen L, Milstein DMJ, et al. Intraoperative Imaging Techniques to Visualize Hepatic (Micro)Perfusion: An Overview. *Eur Surg Res*. 2020;61(1):2-13.
50. Eriksson S, Nilsson J, Lindell G, Sturesson C. Laser speckle contrast imaging for intraoperative assessment of liver microcirculation: a clinical pilot study. *Med Devices (Auckl)*. 2014;7:257-61.

6. Supplementary information

6.1 Organ chamber requirements

The requirements for the organ chamber can be found in Supplementary Table 1.

Supplementary Table 1. *Requirements for the organ chamber.*

Requirement	Description	Test Description	Test setup description	Value	Nice to have	Pilot setup	Prototype	Clinical prototype	Rational
Technical requirements									
Size/envelop	The system must have the dimensions to contain a liver.	Measurement with ruler.	N/A	Max 0.5x0.5x0.5 m	N/A	Yes	Yes	Yes	The system must be big enough to contain a liver, but not bigger than necessary. A big system is more inconvenient and is harder for one person to manage during set up and perfusion.
Material	It must be possible to sterilize the material.	Material characteristics	N/A	Sterilizable with gamma radiation	Steam, formaldehyde, plasma sterilization	Maybe	TBD	Yes	The materials of the system must be sterilizable. In the pilot setup, the system will not be used sterile, so for this stage it is not necessary to have a sterile system. However, it would be useful to use sterilizable material, such that the material does not have to be changed in further stages. The clinical prototype must be sterilizable, so it can be used in a clinical sterile setting.
	Transparency	Visual inspection to see if light passes through the material. Printed text can be put inside the system, test if it can be read through the material.	N/A	Text must be readable through the material	N/A	Yes	Yes	Yes	During perfusion, the liver must be visible for inspection to see if there are any leaks, kinks in the vessels or cannulas, and other changes. It is important that when the perfusion parameters change, e.g., flow, pressure, temperature, the perfusionist is able to see the liver and inspect if anything changed that could have led to the changes in perfusion parameters.
	Toxicity	Material characteristics	N/A	No toxic materials	N/A	Yes	Yes	Yes	No toxic material should be incorporated in the system for organ and patient safety.
Weight	The system must not be to heavy	Scale	N/A	Max 10 kg	N/A	Yes	Yes	Yes	The system must not be too heavy, so one person can carry it and so it does not need a special construction to support it.
Sterility	The system should be as sterile that the liver could be transplanted after 3 days of (sub) normothermic perfusion.	Test if anything from outside the system can get inside.	Color leak test	No visible color fluid leak	Transplantable after 7 days of perfusion, or even several months	Yes	Yes	Yes	To be able to transplant the liver in the recipient after perfusion, it is important to reduce the infection risk to a minimum. During surgery multiple precautions are taken to reduce this risk as much as possible, such as sterile materials, clothing, gloves and wearing surgical masks and caps, and air flow is tightly controlled. Nevertheless, in health care a 100% sterile work environment is not possible. In the clinical practice the recipient will receive antibiotics after surgery and blood cultures are taken to see if there are any infections that have to be treated. As it is not possible to work 100% sterile, and the perfusion itself will not deteriorate when there is an infection, the system does not have to be 100% sterile. Therefore, the requirement is that it should be possible to transplant the liver into the recipient after 3 days of normothermic perfusion.
		Test if anything from inside the system can get outside.	Color leak test	No visible color fluid leak		Yes	Yes	Yes	
		Compare blood cultures from before perfusion and after perfusion. Are there any new bacteria in the system, or is there bacterial growth?	Laboratory Erasmus MC	TBD		No	TBD	Yes	In the pilot setup, porcine livers from the slaughterhouse will be used, which are not sterile. Thus, at this stage the system does not need to be sterile. In the clinical prototype, human research livers will be used, and the system must be sterile to make the analyzation of blood cultures possible and of added value.
Sterility of components	The system should be as sterile that the liver could be transplanted after 3 days of (sub) normothermic perfusion.	Test if fluid from outside the system can get inside.	Color leak test	No visible color fluid leak	Transplantable after 7 days of perfusion, or even several months	Yes	Yes	Yes	Not the entire system has to be sterile. Every surface that comes into contact with the liver or the perfusion solution has to be sterile. Anything that does not come into contact with the liver or perfusion solution does not need to be sterile.
		Test if fluid from inside the system can get outside.	Color leak test	No visible color fluid leak		Yes	Yes	Yes	
		Test if gas from outside the system can get inside.	Vacuum test	No visible gas leak		Yes	Yes	Yes	
		Test if gas from inside the system can get outside.	Water bath test	No visible gas leak		Yes	Yes	Yes	
		Compare blood cultures from before perfusion and after	Laboratory Erasmus MC	TBD		No	TBD	Yes	In the pilot setup, porcine livers from the slaughterhouse will be used, which are not sterile. Thus, at this stage the system does not need to be sterile. In the clinical

Supplementary Table 1. *Continued*

		perfusion. Are there any new bacteria in the system, or is there bacterial growth?							prototype, human research livers will be used, and the system must be sterile to make the analyzation of blood cultures possible and of added value.
Closable	The system should be closable to protect the liver.	Test if fluid from outside the system can get inside.	Color leak test	No visible color fluid leak	N/A	Yes	Yes	Yes	To keep the system and liver sterile, the system must be sealable. When sealing the system during perfusion, nothing from the outside can get into the system: no gas exchange and no fluid exchange.
		Test if fluid from inside the system can get outside.	Color leak test	No visible color fluid leak	N/A	Yes	Yes	Yes	
		Test if gas from outside the system can get inside.	Vacuum test	No visible gas leak	N/A	Yes	Yes	Yes	
		Test if gas from inside the system can get outside.	Water bath test	No visible gas leak	N/A	Yes	Yes	Yes	
Liver accessibility	It must be possible to open and close the system to be able to reach and physically touch the liver and perform small interventions.	Perform 1 opening and closing cycle. During the cycle open the system, touch and maneuver the liver, and close the system.	N/A	Minimum of 20 times opening and closing.	N/A	Yes	Yes	Yes	The user must have access to the liver during the perfusion. It is important to be able to connect the liver to the system, to inspect and touch the liver, to control possible bleedings, to alter the positioning of the cannulas of the blood vessels and the bile duct, if needed to perform the switch from hypothermic to normothermic perfusion and to perform other interventions. The system should therefore be resealable. The minimum of 20 times is based on clinical experience in the Erasmus MC.
		Test if it is possible to connect the liver to the system	N/A	Possible	N/A	Yes	Yes	Yes	
		Test if it is possible to suture	N/A	Possible	N/A	Yes	Yes	Yes	
		Test if it is possible to alter the position of the blood vessel cannulas	N/A	Possible	N/A	Yes	Yes	Yes	
		Test if it is possible to alter the position of the bile duct cannula	N/A	Possible	N/A	Yes	Yes	Yes	
Prevent pressure necrosis	The system must not inflict local pressure necrosis.	Visual inspection of the liver	N/A	No necrotic areas	N/A	Yes	Yes	Yes	In the abdomen, the liver and other organs move with the movement of the diaphragm when breathing. From literature can be concluded that when the liver lays static on a surface, local pressure necrosis will develop at the places of contact. This system should prevent local pressure necrosis by either movement of the liver or submerging the liver in a fluid. Different imaging options can be explored, not all have to be functional. The best options will be used for detecting pressure necrosis.
		Tissue biopsies	Pathology and lab	TBD	N/A	Maybe	Yes	Yes	
		Tissue oxygenation	SFDI	TBD	N/A	No	Maybe	Maybe	
		Tissue perfusion	Laser speckle	TBD	N/A	No	Yes	Yes	
		Fluorescence imaging	ICG, fluorescein	TBD	N/A	No	Yes	Yes	
		Nuclear imaging	FDG-PET/CT	TBD	N/A	No	Maybe	Maybe	
Peripheral perfusion	The system should ensure peripheral perfusion.	Visual inspection of the liver	N/A	Perfused peripheral tissue	N/A	Yes	Yes	Yes	In the abdomen, the movement of the diaphragm induces pressure changes in the abdomen, creating a sponge like effect of the liver. Lower pressures in the abdomen lead to filling of the peripheral vessels, and a higher pressure will lead to a squeeze like effect of the liver.
		Tissue oxygenation	SFDI	TBD	N/A	No	Maybe	Maybe	
		Tissue perfusion	Laser speckle	TBD	N/A	No	Yes	Yes	
		Fluorescence imaging	ICG, fluorescein	TBD	N/A	No	Yes	Yes	
		Nuclear imaging	FDG-PET/CT	TBD	N/A	No	Maybe	Maybe	
Prevent drying out	The liver and structures should not dry out.	During perfusion visual and tactile inspection of the liver every 2 hours.	N/A	Wet or moist structures and liver.	N/A	Yes	Yes	Yes	In the abdomen, the liver is surrounded by other livers and structures. Between these structures there is peritoneal fluid, a serous fluid produced by the peritoneum, which lubricates the surface of tissues in the abdomen. From experience in the Erasmus MC, leaving the liver out in the open, without any

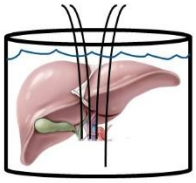
Supplementary Table 1. *Continued*

									surrounding fluid or humidification of the air, the liver and vessels will dry out and become hard. This is something that must be prevented if the liver is going to be transplanted.
Facility	During an open system	N/A	N/A	N/A	N/A	No	Maybe	Yes	When the system is open, the requirements for the facility are the same as the requirements for an OR. In the pilot stage, this will not be necessary, as the porcine livers are not sterile. Once the system is sterile, the system must be opened in a sterile facility.
	During perfusion	N/A	N/A	N/A	N/A	Yes	Yes	Yes	When the system is closed during perfusion, there are no requirements to the facility.
User requirements									
Sterile work environment	Sterile work environment for the user.	Test if it is possible to connect the liver in a sterile manner.	N/A	Possible to stay sterile	N/A	Yes	Yes	Yes	The system must have a sterile work environment for the user. The user should be able to have sterile access to the liver, perform interventions and retrieve the liver from the system for either a switch from hypothermic to normothermic perfusion, or at the end of perfusion.
		Test if it is possible to get to the liver in a sterile manner.	N/A	Possible to stay sterile	N/A	Yes	Yes	Yes	
		Test if small interventions are possible in a sterile manner.	N/A	Possible to stay sterile	N/A	Yes	Yes	Yes	
		Test if retrieving the liver from the system is possible in a sterile manner	N/A	Possible to stay sterile	N/A	Yes	Yes	Yes	
Easy access to the liver	Easy access for the user	Test if it is possible for one user to operate the system both unsterile and sterile.	N/A	Possible to operate	N/A	Yes	Yes	Yes	It should be possible for one user to easily access the liver for minor interventions such as altering the position of the cannulas or suture a leakage and inspection during perfusion.
Perfusion process	Start phase: connecting the liver and start perfusion	N/A	N/A	N/A	N/A	N/A	N/A	N/A	When the liver arrives in the transplantation center, it is first inspected and prepared on the back-table. This procedure is mostly performed by two surgeons, and they prepare the liver for the transplantation and perfusion. Excess tissue and structures are removed, and the cannulas are sewn in. The liver is then placed into the system, and the cannulas are connected to the system air free. The user of the system will then start the perfusion. The surgeon will suture possible leakages and can adjust the positioning of the cannulas to ensure good perfusion of the liver. When the perfusion is stable, the next phase starts.
	Perfusion phase	N/A	N/A	N/A	N/A	N/A	N/A	N/A	During this phase, the perfusion parameters: the flow, pressure, and temperature, are closely monitored and if needed set points can be changed. To monitor the liver, blood gas samples of the perfusate are taken by the user and abnormal values are corrected by administering medication or changing the gas flow and composition. When the perfusion parameters are stable, no interventions are needed, and the system can stay closed. The system can stay closed for medication administration or changes in gas settings. It is possible that small alterations to the cannula position have to be made by the surgeon and the system has to be opened. From clinical experience, repositioning the bile duct cannula is often necessary to start bile flow. In this phase, the system will be closed most of the time.
	End phase: stop perfusion and retrieve the liver from the system	N/A	N/A	N/A	N/A	N/A	N/A	N/A	In the end phase of perfusion, the liver will be disconnected from the system and put on the back-table again. For disconnection, the system needs to be opened and the perfusion stopped. The liver is disconnected by the surgeon, and the system stopped by the user. After warm perfusion, the liver is cooled again with a cold storage solution and after flushing the liver the cannulas are removed. The liver is taken to the OR on ice.

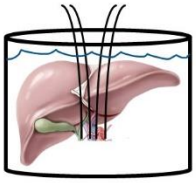
6.2 Organ chamber concepts

The different concepts for the organ chamber can be found in Supplementary figure 1.

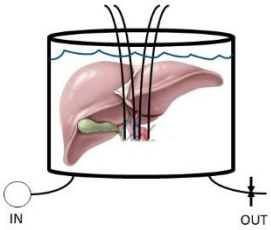
Concept 1
- Liver in a sealable bag with perfusate as the surrounding fluid
- Open perfusion circuit



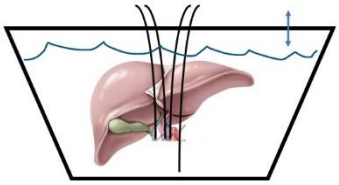
Concept 2
- Liver in a sealable bag filled with fluid surrounding the liver
- Closed perfusion circuit



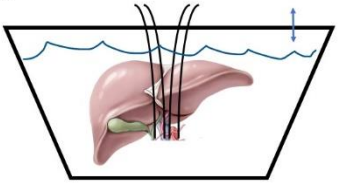
Concept 3
- Liver in a sealable bag filled with fluid surrounding the liver
- In and out flow of the fluid
  - Option to control in and outflow
- Closed perfusion circuit



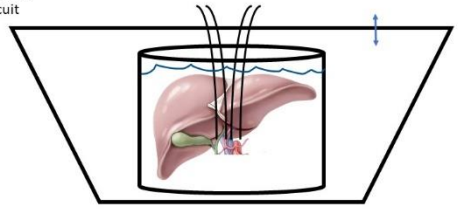
Concept 4
- Liver in a sealable container with perfusate as the surrounding fluid
- Column of air above the fluid which can be varied to change the pressure
- Open perfusion circuit



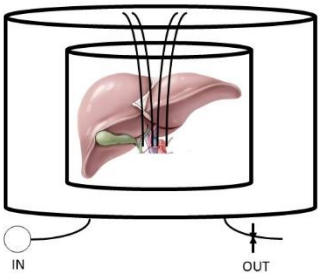
Concept 5
- Liver in a sealable container filled with fluid surrounding the liver
- Column of air above the fluid which can be varied to change the pressure
- Closed perfusion circuit



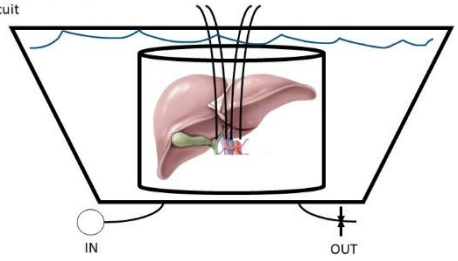
Concept 6
- Liver in a sealable bag filled with fluid surrounding the liver in a container with air
- Amount of air can be varied to change the pressure in the container
- Closed perfusion circuit



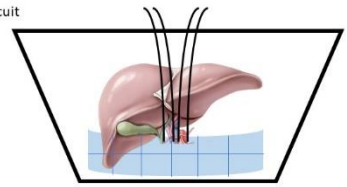
Concept 7
- Liver in a sealable bag where leakage is collected and recirculated to the reservoir
- Outer bag with fluid that flows in and out
  - Option to control in and outflow of the outer bag
- Closed perfusion circuit



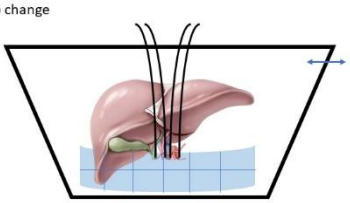
Concept 8
- Liver in a sealable bag where leakage is collected and recirculated to the reservoir
- Amount of water in the container can be varied to change the pressure on the liver
- Closed perfusion circuit



Concept 9
- Liver in a sealable container with a water or air mattress underneath the liver
  - Option to make multiple compartments in the mattress
- Open or closed perfusion circuit



Concept 10
- Liver in a sealable container with a water or air mattress underneath the liver divided in different compartments
- Amount of air can be varied to change the pressure in the container
- Open or closed perfusion circuit



Supplementary figure 1. *Ten organ chamber concepts.*

6.3 LiMAx score calculation

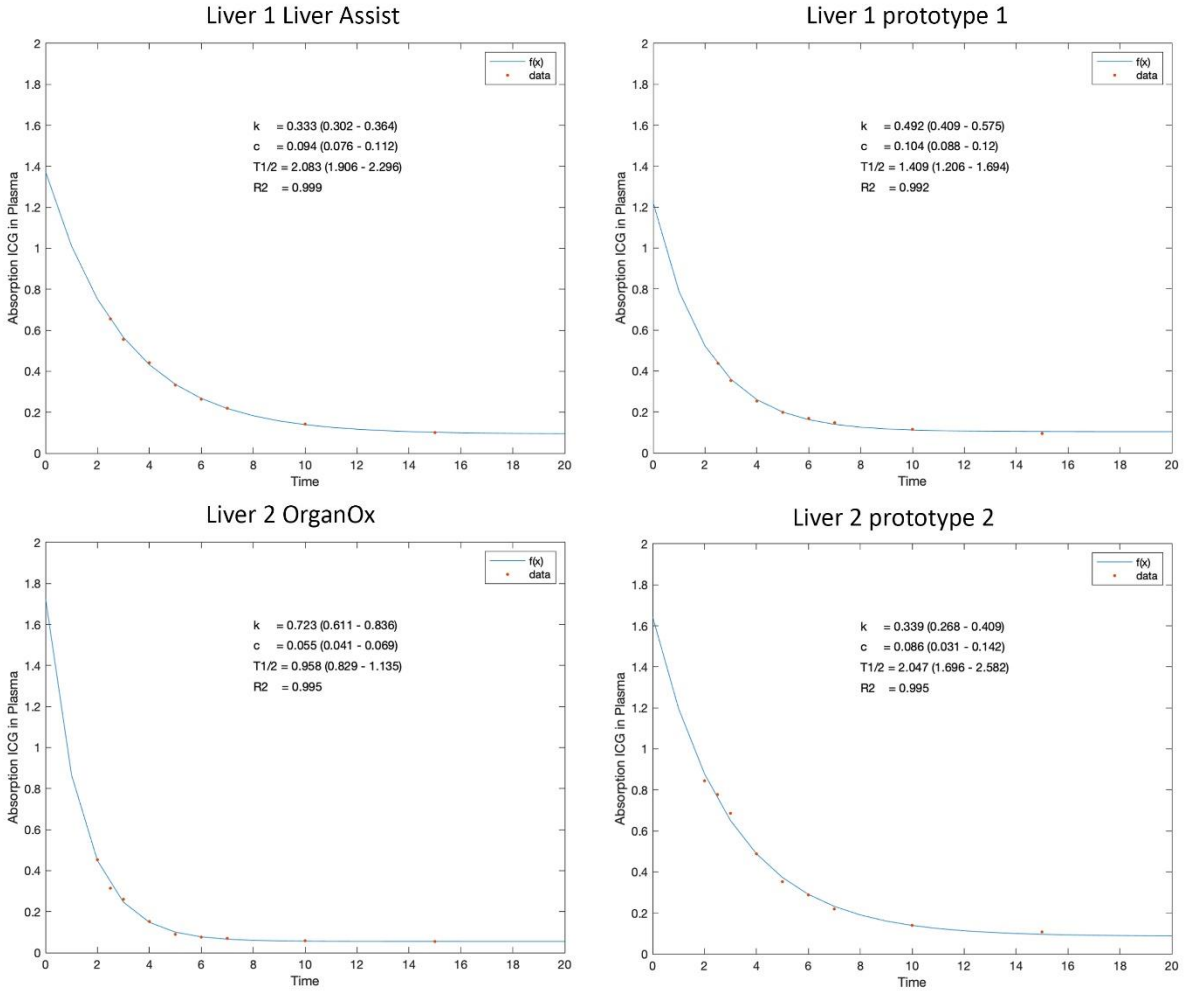
The LiMAx values were calculated using the following formula:

$$LiMAx = \frac{DoB_{max} * \mathrm{C} * \mathrm{P} * \mathrm{M}}{\frac{1}{2}D} \qquad \text{(Formula 2)}$$

The LiMAx values are calculated in µg/kg/h. DoB_{max} represents the maximum of the DoB kinetics, C is a constant (0.011237), P represents the estimated production rate of CO_2 (300 mmol/h) per body surface (m^2) corrected for the liver's estimated use of 17.52% of the resting energy consumption, M is a constant of the molecular mass of ^{13}C-methacetin (166.19 g/mol) and lastly, D is the dosage of ^{13}C-methacetin.

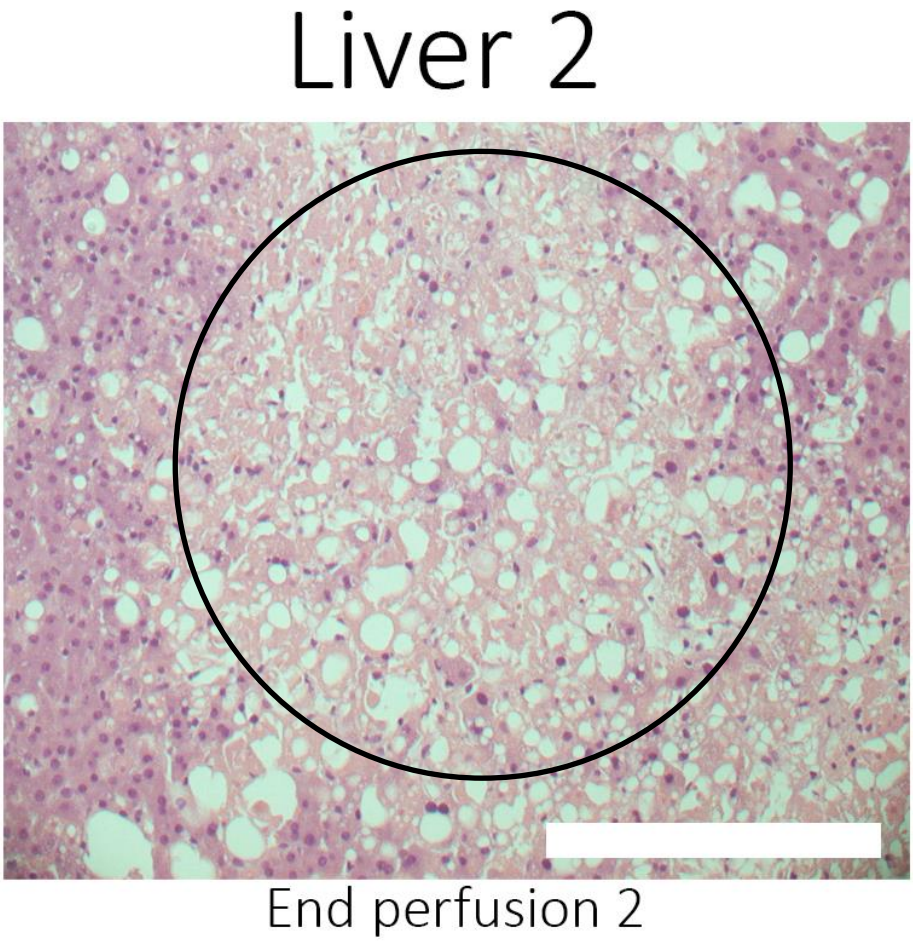
6.4 ICG absorption graphs

The ICG absorption graphs from all four perfusions are shown in Supplementary figure 2.



Supplementary figure 2. *ICG absorption graphs of all four perfusions.*

6.5 Histology



Supplementary figure 3. *Necrotic area indicated by the circle after 2 perfusions in liver 2. All scale bars represent 100 µm.*