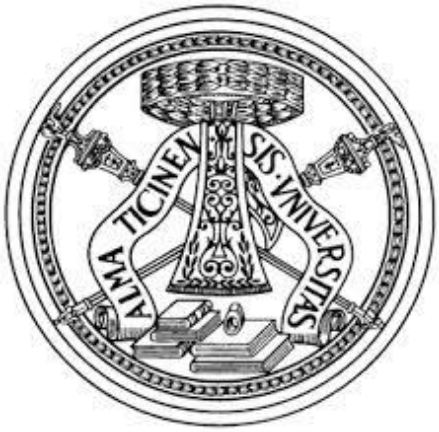

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Ischemic reperfusion injury in Liver transplantation

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TABLE OF CONTENT:

ABSTRACT:

About 119,592 patients are currently on the organ transplant waiting list in the US, with the number increasing by 5% every year. In 2017, 11,640 candidates were added to the liver transplant waiting list. While the increase in the number of liver transplants is encouraging, the organ shortage remains critically high. During 2015, 1673 patients died without undergoing transplant and another 1227 were removed from the waiting list due to being too sick to undergo transplant.

The major untapped pool of donor organs that could be used to alleviate this crisis in organ transplantation are steatotic livers and livers from Donors after Cardiac Death (DCD). Steatotic liver grafts are associated with an early allograft dysfunction rate of 60%-80% compared with less than 5% for nonsteatotic grafts. This is due to their poor tolerance to ischemic reperfusion injury. On the other hand, only 27% (518/1884) of the DCD livers were transplanted in 2017. The single major reason for this is the severe ischemia reperfusion injury in these livers.

The severity of ischemic reperfusion injury is an important determinant of allograft function post-transplant. However, the mechanisms that contribute to increased susceptibility of steatotic and DCD grafts to ischemic reperfusion injury remains poorly defined. In solid organ transplantation, graft damage subsequent to ischemic reperfusion injury may result in delayed graft function. In the worst case, this complication can lead to primary graft non- function resulting in an urgent need of re-transplantation.

Ischemic reperfusion injury is the consequence of temporary interruption of blood flow to the liver. Warm ischemia reperfusion injury occurs during clamping of vascular inflow during prolonged liver resections and during a donation after cardiac death organ retrieval procedure. Cold ischemia reperfusion injury results from maintenance of the liver in cold preservation and subsequent reperfusion of the graft during transplantation. Apart from its pivotal role in the pathogenesis of the liver's post reperfusion injury, it has also been involved as an underlying mechanism responsible for the dysfunction and injury of other organs as well. Liver ischemia and reperfusion in settings of liver transplant represent an event with consequences that influence the function of many organs including the lung, kidney, intestine, pancreas, adrenals, and myocardium among others. The molecular and clinical manifestation of these remote organ injuries can ultimately lead to multiple organ dysfunction syndrome.

The objective of this thesis is to give a full and comprehensive analysis of ischemic reperfusion injury in liver transplantation.

In the first chapter, I will provide a full description of the etiology and pathophysiology of ischemic reperfusion injury. I will address specifically the pivotal role of hepatocyte's mitochondria, oxidative stress, role of cytokines and other bioactive molecules.

In the second chapter, I will present the role of ischemic reperfusion injury in settings of liver transplantation and overall literature review. Here, it will be addressed the impact of ischemic reperfusion injury in regular and marginal

(steatotic and donor after cardiac death) liver. Outcomes such as graft and patient survival will also be discussed.

Chapter three will be dedicated to my personal work and experience at Indiana University Purdue University Indianapolis (IUPUI). It will be divided between my clinical work study (OrganOx trial) and lab work on mice and rats. Data will be presented, describing ischemic reperfusion injury and its impact on both human and animal livers.

In the last two chapters, I will discuss our findings and how they can be applied in clinical work, as well as identifying future prospective in the field of ischemic reperfusion injury.

LIST OF MOST COMMON ABBREVIATIONS:

IRI – Ischemic/ischemia reperfusion Injury

OLT – Orthotopic liver transplantation

ALT – Alanin transferasis

AST – Aspartam transferasis

GGT – Gamaglutamil transferasis

ATP - Adenosine triphosphate

ROS - Reactive oxygen species

DAMPs - Damage-associated molecular patterns

NMP – Normothermic machine perfusion

HMP – Hypothermic machine perfusion

DCD- Donation after Cardiac Death

GLOSSARY:

Ischemic/ischemia reperfusion injury

Damage that occurs in tissue caused when blood supply returns to tissue after a period of ischemia/lack of oxygen

Piggy – back technique

Surgical technique used in liver transplantation, where the native liver is removed without using venous by-pass.

Normothermic machine

Device where the explanted organ is reperfused with the donor's or external blood after being harvested.

Hypothermic machine

Device the explanted organ is reperfused with cold oxygenated perfusate.

CHAPTER I:

Ischemic reperfusion injury:

etiology, cell biology and pathophysiology

1. **Etiology of Ischemic reperfusion injury:**

Ischemia reperfusion injury (IRI) is a major cause of morbidity and mortality in liver transplantation surgery. Prolonged organ ischemia is characterized by reduced tissue oxygenation resulting in tissue adenosine triphosphate (ATP) depletion. This transitions to an activation of anaerobic metabolic pathways which cannot maintain cellular function for prolonged periods ultimately leading to cell death. Restoration of blood flow is necessary to restore cellular function, but paradoxically reperfusion can initiate a cascade of pathways that cause further cellular injury after even a short period of ischemia. Understanding the mechanisms of liver ischemic reperfusion injury and developing strategies to counteract this injury will reduce acute complications in liver transplantation, ultimately expanding the potential pool of usable donor grafts. The IRI can be classified as warm (normothermic) or cold (hypothermic). The first form occurs during liver surgery, trauma and shock. The latter form occurs during the preservation and storage of the organ for transplant.

Ischemia induces a variety of cellular metabolic and ultrastructural changes promoting expression of proinflammatory gene products including cytokines, circulating chemokines

and reactive oxygen species (ROS) while repressing protective gene products [1]. Thus, ischemia induces a proinflammatory state that increases tissue vulnerability to further injury on reperfusion.

The mechanisms of hepatic IRI have been widely investigated, but nevertheless remain largely unclear. The complexity in mechanisms and cellular components implicated in IRI has led to several controversies, and even discrepancies in our understanding of this pathology.

2. Mechanisms of injury

2.1 *Role of hepatocyte and sinusoidal injuries*

The most important factor is hepatic endothelium damage occurring during cold preservation. This represents the initial factor leading to hepatic IRI injury, determining poor graft microcirculation, platelet activation, persistent vasoconstriction, up-regulation of adhesion molecules, cytokine release, oxidative stress, Kupffer cell activation, neutrophil infiltration and hepatocyte death, thus contributing to the development of primary non-function or impaired primary function after liver transplantation. Although hepatocyte function and viability might be preserved under *in vitro* cold storage conditions up to 72 h, the liver sinusoidal endothelial cells phenotype is rapidly

deregulated during cold storage, leading to cell apoptosis (2). Another integral and crucial part of liver damage impact liver sinusoidal endothelial cells (LSEC). Those cells during cold storage have been compromised. The lack of biomechanical stimuli occurring during cold preservation for transplantation markedly deteriorates the

LSEC protective phenotype by down-regulating the expression of the transcription factor KLF2 (Kruppel-like factor 2), which orchestrates the transcription of a variety of protective genes including the eNOS (endothelial nitric oxide synthase), the anti-thrombotic molecule thrombomodulin or the antioxidant transcription factor Nrf2 (nuclear factor-erythroid 2-related factor) (3). Concomitantly to LSEC deregulation, Kupffer cells suffer from a profound activation process that is promoted by the release

of DAMPs (damage-associated molecular patterns) from adjacent necrotic hepatic cells.

2.2 *Role of oxidative stress and mitochondrial damage*

Oxygen is critical for cellular existence and oxygen homeostasis is fundamental to human physiology (4). The reduction of oxygen to water in the mitochondrial transport chain is crucial in maintaining the oxygen homeostasis and supplies the metabolic demands with ATP. During hepatic ischemia, the metabolic pattern is shifted from aerobic to anaerobic and all metabolic activities are gradually stopped. In these conditions of ATP depletion, increases lactate formation, and alterations on cellular PH homeostasis, thus inducing important damages on the hepatocyte (5). Ischemia also leads to a considerable increase of DAMPs and as a result leads to the phosphorylation/deregulation of key enzymes involved in the control of carbohydrate metabolism (6). Consequently, an accumulation of acidic metabolites may occur, such as lactic acid and ketone bodies accompanied by hypofunction of mitochondrial oxidative phosphorylation, resulting in the decrease of pH values

between tissues and cells. When an ischemic liver is revascularized, the generation of numerous reactive oxygen species (ROS) and reactive nitrogen species (RNS) is occurring. These radicals act on different cell's proteins, enzymes, nucleic acids and lipid peroxides, leading to cell damage and in particularly to mitochondrial dysfunction and lipid peroxidation (7). In addition, ROS and RNS could potentially damage endothelial cells and destroy the integrity of the microvasculature. Under normal circumstances ROS and RNS are neutralized through diverse antioxidant mechanisms present in the cell. Unfortunately, under stress conditions, like IRI, the balance between ROS and antioxidants shifts towards the former, resulting in oxidative stress and cytotoxicity (8). The primary effect of ROS during IRI are into transformation of xanthine dehydrogenase into xanthine oxidase (an oxygen dependent process that produces uric acid, releasing the ROS superoxide and hydrogen peroxide), induction of NADPH oxidase and nitric oxide (NO) production and its conversion to peroxynitrite (both considered reactive nitrogen species) (9). Within the liver, the cytotoxic effects of ROS translate into nitrosylation of iron-sulfur groups and tyrosine residues, inactivation of the heme group, and lipid peroxidation (10). Another important characteristic of ROS is their pivotal role in the inflammation response after reperfusion. This response can be divided in two phases - an acute (initial) phase, and a sub-acute phase (11). The acute phase corresponds to the first 4 to 6 hours following reperfusion. In this stage mitochondrial ROS are essential for the activation of the Kuppfer cells, which later will mediate the release of proinflammatory cytokines and chemokines (12). The sub-acute phase is the inflammatory stage, characterized by a massive infiltration of neutrophils, release of cytotoxic and proinflammatory mediators, and activation

of the mesenchymal stem cells which will lead to post-stress fibrosis. During the late stage, the main source of ROS is the mitochondrial NADPH oxidase enzyme (NOX 3,4 and 5) which work as amplifiers of the

post-stress inflammatory response (13). At the molecular level, elevated ROS levels boost the inflammatory cascade, partially through the direct activation of the transcription

factor NF-κB, and other indirect mechanisms which will promote a pro-inflammatory status

increasing the levels/activity of TGF-β, TNFα and IL-1ß and other cytokines (14). ROS is a crucial mediator of ischemic preconditioning. ROS trigger many cellular responses that contribute to ischemic preconditioning. For example, ROS facilitate the activation of adenosine monophosphate-activated protein kinase (AMPK) and stabilization of the transcription factor HIF-1α, resulting in a shift in the cellular metabolism, making the liver more glycolytic and less dependent on oxygen. This compensatory effect facilitates survival immediately following reperfusion, since during reperfusion not all the tissue recovers immediately, normal oxygen tension and capillaries tend to collapse. Recovery of normal tissue perfusion depends on the formation of new microvessels, which is tidily related with HIF-1α dependent activation of the angiogenesis mediator VEGF-A (15).

The amount of ROS created during IRI is related with the tissue levels of antioxidants. In fact, reduced levels of the antioxidant proteins have been associated with larger cell damage, while forced increase of antioxidants has the opposite effect (16). Another important role of ROS is that it can result in the induction of antioxidant systems through the activation of the transcription factors

- nuclear factor erythroid 2-related factor 2 (Nrf2) (17) and the transcriptional coactivator PGC-1α. Both have been shown to be induced in response to oxidative stress and increase the expression of antioxidant levels in hepatocytes (18,19). It is important to know that, while the normal liver responds to IRI inducing PGC-1α and antioxidant gene expression, the steatotic liver shows a significant reduction in its capacity to induce antioxidant gene expression in response to IRI. Therefore, downregulation of the transcriptional co-activator PGC-1α and Nrf2 in the steatotic liver limits its capacity to control antioxidant enzyme levels and to modulate them in response to IRI. In the healthy liver, in response to IRI,
PGC-1α levels are induced and this induction results in the augmentation of the cellular antioxidant defenses.
At the end - the picture that emerges is that ROS play a double edged role in IRI - on one hand promotes apoptotic cell death and induces inflammatory mediators, but on the other hand, facilitates cell survival in hypoxic conditions and induces antioxidant defenses.

2.3 *Role of immune response and neutrophils*

The liver damage is caused during the reperfusion period, when there is a shift from metabolic distress caused by ischemia to an excessive immune response triggered by reperfusion. Liver IRI shows a significant inflammatory component, and neutrophils are playing central role in the liver damage occurring after reperfusion (20). During IRI excessive numbers of neutrophils are recruited and activated and

this excessive neutrophil influx contributes to the pathogenesis of IRI. This IRI-induced liver injury is a multistep process that starts with neutrophil activation, going through trans endothelial neutrophil migration, and finish with contact to parenchymal liver cells. In this process are involved different types of cells such as leukocytes, including natural killer cells, natural killer T cells, dendritic cells, neutrophils, eosinophils, and complement components (21). Of these cell subsets, neutrophils are known to

induce liver injury and are the largest circulating fraction of leukocytes. Neutrophils are the first cells to arrive at the site of injury. During IRI, neutrophils migrate intravascularly through the sinusoid channels toward the stressed tissue, ultimately infiltrating directly into the area of damage. The oxidative stress during liver IRI results in the passive release of multiple danger-associated molecular patterns (DAMPs) from necrotic cells. DAMPs are group of different molecules including high mobility group box 1 (HMGB1), deoxyribonucleic acid (DNA), ATP, urate, mitochondrial formyl peptides, and S100 proteins. Those molecules induce sterile inflammation through stimulation of chemokine production by resident cells and, consequently, neutrophil recruitment. In the animal model, purified DAMPs mobilize neutrophils to the site of inoculation (22). Another important mediator in IRI is the mitochondrial content which is spilled out into the extracellular matrix during liver reperfusion. This occurs mainly from mitochondrial N-formyl peptides that attract and activate neutrophils through the specific receptors on their membrane. Neutrophil-derived protease is another factor that seems to have an important role in the cause of direct cell death of hepatocytes. Once neutrophils adhere to hepatocytes, full degranulation occurs, with consequent release into the

area around the cell of several proteases, such elastases, cathepsin G, and proteinase-3 (23). Neutrophil elastase is a serine protease found in the azurophil granules of all neutrophils. This enzyme is involved in the pathogenesis of inflammatory tissue injury such as that exemplified by liver IRI. In the context of liver IRI, there is overwhelming evidence for a deleterious role of neutrophil migration and function, and the activity of the neutrophil proteases together with ROS production appears to mediate most of the deleterious actions of neutrophils.

2.4 Role of the Intracellular calcium overload

Another pivotal factor in the pathophysiology of IRI is the intra hepatocyte calcium level. Immediately after reflowing of the hepatic blood, hepatic calcium increased rapidly, with a peak value at 30 minutes after reperfusion. Changes in cytoplasmic Ca (Ca_{cyt}) and in the concentration of Ca in intracellular organelles play essential roles in the regulation of normal hepatocyte function in response to different extracellular signals (24). The abrupt change in Ca_{cyt} comes through two main paths - one is calcium influx into viable hepatocytes, and the other is calcium accumulation in dead liver cells. Generally, the hepatocyte's cell membrane has a potent function of pumping out calcium against a l0 000-fold gradient between the intracellular and extra- cellular space. This action is mainly maintained by Ca ATPase and Na + exchange systems which use energy. Some evidence has been obtained to

indicate that the IRI-induced increases in total Ca in the intact liver and increases in Ca_{cyt} in hepatocytes are due to enhanced Ca inflow across the plasma membrane (26). Although the Ca-permeable channels responsible for this Ca inflow have not yet been identified, studies have shown that ROS activate a 16 pS nonselective cation channel which could admit Ca (27). In addition, different studies have identified several Ca-permeable channels which are activated by ROS (28). The excessive Ca_{cyt} will promote activation of calcium-dependent autolytic enzymes such as phospholipase, protease and nuclease, resulting in mitochondrial hepatocyte injury, which leads to cell death. (25). Moreover, enzymes regulated by Ca_{cyt} may also fail. These factors will promote liver cell injury and lead to cell death after reperfusion. Those findings are confirmed by experiments conducted with isolated perfused animal livers, where the reperfusion after ischemia leads to an increase in the total amount of Ca in hepatocytes and subsequent hepatocyte injury.

2.5 Role of Endoplasmic reticulum stress response

The endoplasmic reticulum (ER) is an important cell organelle involved in calcium homeostasis, protein folding and lipid biosynthesis. Perturbations in its normal functions lead to a condition called endoplasmic reticulum stress. This can be triggered by many physiopathological conditions such as ischemic reperfusion injury. Under normal situations, a homeostatic equilibrium exists between the influx of unfolded peptides and the folding ability of the ER. Once under stress ER

activates intracellular signal transduction pathways which together are known as the unfolded protein response (UPR) (29). It is known that hepatocytes are the main place for synthesis of protein and lipids. As the result of these activities, they contain abundant quantities of rough and smooth ER. Hence, during IRI, ER homeostasis is impaired, new unfolded proteins are accumulated and UPR is activated. This occurs during cold storage of the liver graft. UPR alteration is then increased after reperfusion, which is determinant for the graft outcome after transplantation. Unfolded protein response is activated upon ER stress during IRI and three ER transmembrane receptors: PREK, ATF6 and IRE1, transduce signaling cascade to inhibit new protein synthesis and activate transcriptions of selective sets of gene encoding proteins involved in protein folding and protein degradation in ER (30). If UPR fails to resolve ER stress or the stress is prolonged, pathological outcomes will follow, including cell death and inflammation. It is clear that cells utilize multiple mechanisms to cope with ER stress (31). Autophagy is the main one. Autophagy is an evolutionarily conserved and lysosome-dependent system for degradation and recycling of proteins, organelles and other cellular components (32, 33) damaged after significant stress event, such as IRI. As a cell response, autophagy is able to efficiently remove damaged organelles and protein aggregates, and serve as a safeguard when UPR fails. Autophagy is normally active in most cells for optimal maintenance of homeostasis. Autophagy can either protect cells from necrotic/apoptotic death or promote cell death via autophagic cell death pathway. Both detrimental and beneficial effects of autophagy inhibition have been reported on the development of IRI. IRI can either increase or decrease autophagy activities depending on affected organs and/or ischemia time. In fact, in

settings of liver transplantation where the ischemia time is long, there is marked increase of hepatocyte death due to autophagy.

In summary, there is a functional relationship between ER stress and autophagy in the pathogenesis of liver IRI. The functional dichotomy of stress responses, dictated by the duration/severity of ischemia, is dependent on their interactions. Autophagy activity is a key determinant of the outcome of stress responses in the IRI process.

2.6 Role of nitric oxide

The role of nitric oxide (NO) in IRI is complicated and still inconclusive. NO is an unstable carbon-centered radical with a short half-life. There are two sources: one is non-enzyme derived and other main one, which is enzyme induced, in which NO is produced in a redox reaction between L-arginine and oxygen molecules by NO synthase (NOS) catalyzation. There are three types of NOS: endothelial nitric oxide synthase (eNOS) found in all endothelial cells, neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) found in in the cytoplasm of some inflammatory cells. NO is produced mainly by eNOS catalysis, and in a small part by the upregulation of iNOS expressions during acute hepatic ischemia (34). NO was proven to reduce hepatic IRI through various mechanisms (35, 36), such as inhibiting liver cell apoptosis, slowing the infiltration of macrophages, eliminating superoxide anion produced by neutrophils, protecting the liver sinus structure and maintaining liver microcirculation blood flow, accelerating the liver tissue oxygenation, stabilizing ATP levels, decreasing oxidative stress injury, preventing the reduction of glutathione and the increase of endothelin side effects, and

inhibiting platelet aggregation. One of the most important function on NO during IRI is to decrease p53 gene expression and the levels of IL-1 and TNF-α as well as inhibit cell apoptosis to protect the heart, liver, lungs, and kidneys. Elevated NO levels also inhibit p53 gene expression and decrease the production of proinflammatory cytokines and chemokines.

NO also can inhibit the oxidation of mitochondrial cytochrome and reduce ROS production. Excessive ROS is generated in liver cells after its hypoxia/reoxygenation, which causes protein oxidation and lipid peroxidation. Hence, NO reduces ROS production by inhibiting mitochondrial respiratory chain complexes (37) and diminish their damage to hepatocyte.

NO activate soluble guanylyl cyclase (sGC), catalyzing guanosine triphosphate (GTP) to produce cyclic 3', 5' guanosine monophosphate (cGMP). The protection effect of cGMP on hepatocytes is conduct throughout activation of vacuolar H^+-ATPases, which lead to the extrusion of [H^+] from the cytosol of hepatocytes into the extracellular environment, thus keeping cell's pH stable (38).

NO is also an important molecule that is involved in immune regulation. NO inhibits proinflammatory cytokines, including TNF-α, IL-1β, IL-1α, and IL-12, which are involved inducing the inflammatory cascade during IRI. In addition, NO can decrease the number of T helper 1 cells and promote the proliferation of Th2 cells, regulate leukocyte adhesion, and induce the generation of T regulatory cells (39, 40).

NO can have also an opposite effect on hepatocytes during IRI. It has been reported that excessive NO may paradoxically damage liver tissue by forming nitrogen

peroxide, indicating that the dose of NO produced is vital during IRI. This over production of NO is derived by upregulated iNOS in hepatocytes.

In conclusion, NO plays a complicated role during IRI. It is the main way of hepatocytes to contrast the damage on cells provoked by IRI, but on the other hand, if produced in excess can potentially lead to severe cell damage and subsequent apoptosis.

2.7 Role of cytokines and chemokines

Cytokines are a broad category of small proteins which include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors. Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells and fibroblasts (Fig.1). They act through receptors, and are especially important in the immune system; cytokines modulate the balance between humoral and cell-based immune responses, and they regulate the maturation, growth, and responsiveness of particular cell populations. Some cytokines enhance or inhibit the action of other cytokines in complex ways.

Chemokines constitute a group of small structurally related chemotactic proteins indispensable for the coordination of leukocyte migration during inflammation (41). They are potent chemo-attractants for neutrophils and have been shown to contribute to hepatic neutrophil recruitment and liver IRI (42, 43).

Chemokine and cytokine production only is not sufficient to lead to neutrophil recruitment. For chemokines to have an effect on leukocyte trafficking, besides

being produced, they need to be present at a defined concentration range and in the right place to be able to induce chemotaxis. This process requires interaction of chemokines with glycosaminoglycans (GAGs), expressed on the liver vasculature (45). Endothelial cells present GAGs such as heparan sulfate on their cell surface to which chemokines bind via their GAG-binding site. Because of the chemokine–GAG interaction, a stable chemokine gradient is formed on the surface of the endothelial cells and activated neutrophils migrate along this gradient toward the site of inflammation (46). Chemokine binding to GAGs has been proven to be indispensable for chemokine activity and neutrophil recruitment *in vivo* (47, 48). The local concentration of chemokines induces crawling of neutrophils toward an inflammation site. Chemokine production at sites of inflammation also results in the generation of GAG-mediated chemokine gradients in the liver extracellular matrix. Different studies show that the blockade of GAG–chemokine interactions is able to inhibit neutrophil extravasation in a murine model (49). Importantly, removing hyaluronan from the sinusoidal endothelium, or blocking its interaction with its principal receptor (CD44), reduces neutrophil recruitment as well (50). It has been suggested that interruption of chemokine–GAG interactions might represent an innovative and useful way of interfering with chemokine action that may result in a decrease in inflammation (50). After IRI the complicated cytokine synthesis starts with the upregulation of Interleukin 12 (IL-12) and IL-23. Both have been observed to rise significantly minutes after reperfusion and disappeared within 4h (44). Their source is Kupffer cells and hepatic stellate cells. Their principal role is leukotaxis and they boost the level of TNF-α and IFN-γ. Therefore, IL-12 and IL-23 appear to be early response cytokines that amplify the inflammatory response

by stimulating the expression of TNF-α and IFN-γ. One of the most important cytokines involved in IRI is TNF-α (51). TNF-α is released by a variety of cells in the liver, but mostly by Kupffer cells, and it is detected rapidly after reperfusion (52). TNF-α stimulates hepatocytes and Kupffer cells to produce neutrophil chemoattractants, particularly chemokines (53). In addition, TNF-α upregulates different adhesion molecules such as ICAM-1, VCAM-1, on vascular endothelial cells and promoting leukotaxis (54).

Some of the cytokines are anti-inflammatory mediators and have been shown to be expressed after IRI to play key roles in the resolution of the injury response. From this group most prominent are IL -6 and IL -13. IL -6 is a multifunctional cytokine produced by Kupffer cells and macrophages and is released during IRI. It is found out that therapeutic treatment with IL-6 reduced IRI injury. These effects were associated with reduced expression of TNF-α and P-selectin (55). In addition, IL-6 enhanced the activation of signal transducer and activator of transcription 3 (both part of the repair system of the liver) and lead to hepatocyte proliferation after IRI injury (56). Thus, IL-6 appears to function as a factor that resolves inflammatory injury and promotes repair and regeneration. IL-13 is an anti-inflammatory cytokine that limits inflammation by inhibitory effects on the transcription factor NF-κB. IL-13 protected hepatocytes from H_2O_2-induced cytotoxicity. IL-13 therefore appears to have prominent protective effects on hepatocytes and liver endothelial cells.

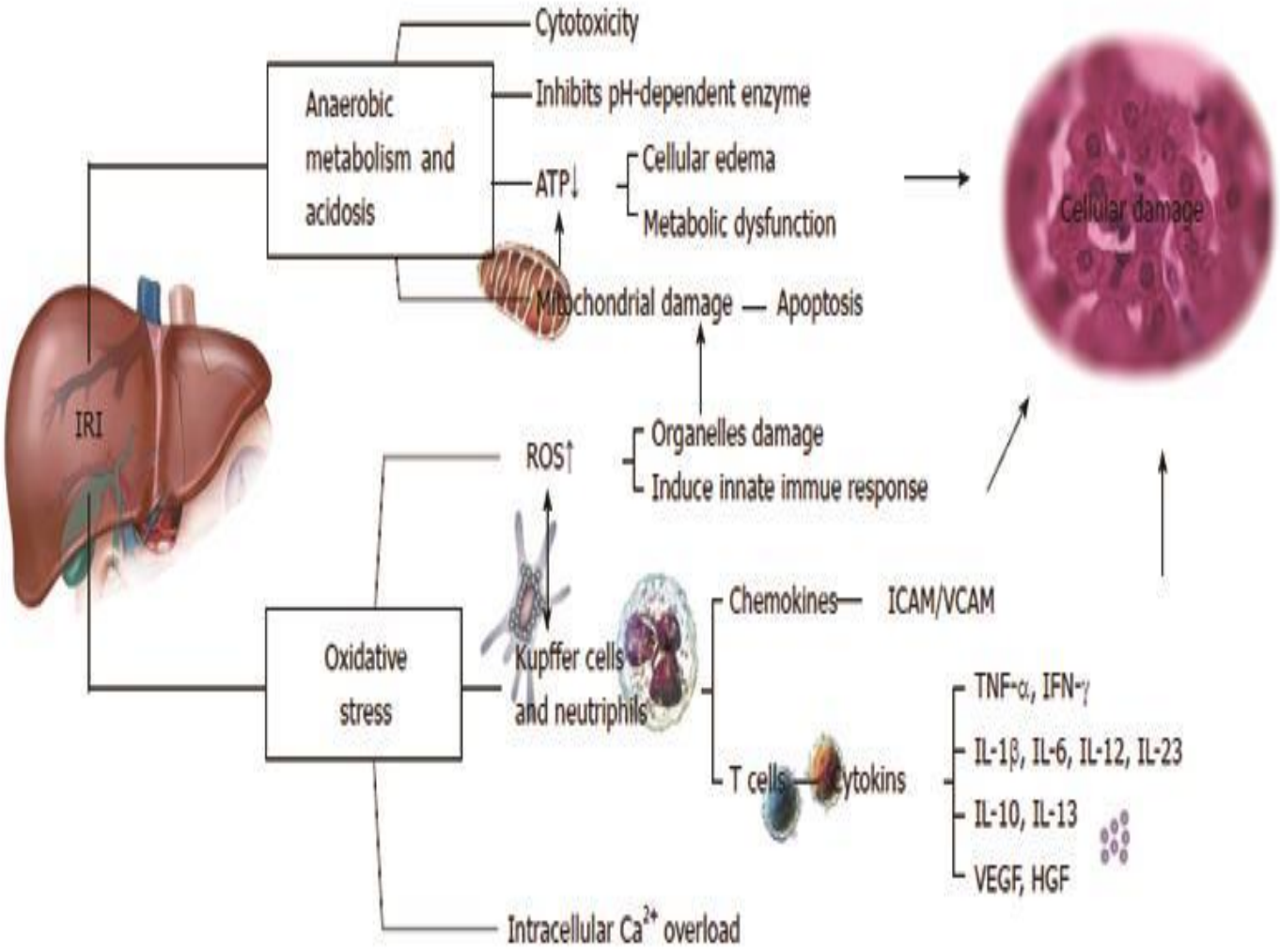
The role of other important cytokines in liver IRI is summarized in Table 1

TABLE 1 ROLE OF OTHER IMPORTANT CYTOKINES IN LIVER IRI

Cytokine	Cellular Source	Effect on liver in settings of IRI	Reference
IFN-γ	T lymphocytes, NKT cells, hepatocytes	increase hepatic IRI	57
Hepatocyte growth factor	Kupffer cells, Ito cells	Reduce hepatic IRI	58
IL-1β	Kupffer cells	Increase hepatic IRI	59
IL-10	Kupffer cells, T lymphocytes	Reduce hepatic IRI	60
IL-6	Kupffer cells	Reduce hepatic IRI	61
IL-12	Hepatocytes, Kupffer cells	Reduce Or Increase hepatic IRI	62
IL-13	Kupffer cells, T lymphocytes	Reduce hepatic IRI	63
IL -18	Kupffer cells	Increase hepatic IRI	64
IL-23	IL-23	Reduce Or Increase hepatic IRI	65
VEGF	Kupffer cells, T lymphocytes,	Increase hepatic IRI	66

Conclusion: In the setting of IRI, cytokines and chemokines play a complex role since they can be a proinflammatory or an anti-inflammatory agent. This complex communication is controlled by the immune and nonimmune systems and their interaction with the liver. Our understanding of these processes remains incomplete and the exit of it is hard to predict.

FIG.1 CUMULATIVE REPRESENTATION OF DIFFERENT FACTORS INVOLVED IN ISCHEMIA REPERFUSION INJURY AND SUBSEQUENT LIVER DAMAGE.



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CHAPTER II:

Ischemic reperfusion injury in liver transplantation and literature review.

1. Global impact of IRI on liver functionality after OLT

Liver transplantation is the only therapy for all patients with end-stage liver disease. However, the waiting list for liver transplantation is growing constantly in all countries, whereas the number of available organs is not growing at a proportional rate. Orthotopic liver transplantation (OLT) dates back to 1963, when Thomas Starzl carried out the first

transplant on a child suffering from biliary atresia. Every single liver transplant comes with one major inherent issue – ischemic reperfusion injury. It is the main cause of initial poor function and primary non-function of the liver allograft. The last one is responsible for up to 81% of re-transplantations during the first week after surgery (1, 2). IRI in the OLT setting is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery (3, 4). In the liver, this form of injury was recognized as a clinically important pathological disorder by Toledo in 1975 during studies of experimental OLT. However, it was not until the mid-1980s that the term reperfusion injury was generally used in the literature on OLT. A variety of clinical factors including starvation of the donor (poor nutrition during the hospital/ICU stay), graft age, steatosis, ICU stay, hypotensive episodes during organ procurement, serology of the donor (current or previous hepatitis infection etc.) contribute to enhanced liver susceptibility to IRI.

In all western countries the waiting list of liver transplantation is growing every year, meanwhile procured organs remain more or less the same. Another important factor is the rise of the death percentage of patients on the liver waiting list. It is estimated that for US between 10% - 22% of these patients will die waiting for a liver transplant. This shortage of organs has led multiple centers to expand their criteria for the acceptance of organs with some “issues” or marginal grafts. One common feature for all marginal liver grafts is that they all show poor tolerance to IRI, which subsequently will lead to graft loss, primary graft non function or patient death. Marginal grafts include: all donor after cardiac death organs (DCD), all donors with age> 70 years, livers with high content of fat (steatotic), grafts with ongoing Hepatitis C (HCV) infection, small for size liver grafts etc.

As described in Chapter I, a large number of factors and mediators play a role in liver IRI. Their relationship between them and the host cells is extremely complex and it is not yet possible to describe with absolute certainty. In a few words the absence of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results
in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. Liver sinusoidal endothelial cells and hepatocytes are targets of IRI-induced cell death. Early IRI-induced cell death is a result of metabolic disturbances and ATP depletion. Following reperfusion, neutrophils and macrophages are activated and accumulate in the liver. These cells exacerbate IRI

through secretion of different molecule signals such as reactive oxygen species and inflammatory cytokines and chemokines. Finally, hepatic stellate cells and hepatocytes become activated in IRI and promote long-term recovery from IRI, which can manifest as full recovery or allograft fibrosis (5).The severity of hepatocyte damage depends on the length of time the ischemia lasts. In OLT, a long ischemic period is a predicting factor for post-transplantation graft dysfunction. It is well established the life of liver graft in static cold preservation, which is up to 10/12 hours. This time is highly variable and depends upon the quality of the graft. In marginal livers this time decreased to almost a half. In short, hepatic IRI worsens the survival of patients needing liver transplants. It reduces the pool of organs available for transplant as some may suffer severe IRI if used. Those patients who experience severe IRI in their allografts have poor graft function and survival after liver transplantation.

2. Clinical context of ischemic reperfusion injury in liver transplantation

2.1 Recipient factors

The risks of IRI in OLT are present in the transplant recipient as well as in the organ donor. Transplant recipients suffer from chronic liver injury (cirrhosis). They demonstrate altered coagulation profiles. Clinically, cirrhosis leads to increased rates of deep venous and/or portal vein thrombosis. The pro-thrombotic profile of cirrhotics increases the risk of IRI before and after liver transplantation; up to 30% of cirrhotics may have thrombosis of the portal vein.

This may cause the patient awaiting a liver transplant to have IRI in their native liver, thus priming them for more severe IRI after liver transplantation (6). In the post-operative period, this pro-thrombotic state is a risk factor for thrombosis of vessels in the newly transplanted liver. The mechanisms of this pro-thrombotic state are multifactorial. Part of the problem is that the blood flow in cirrhotics is more turbulent because of increased resistance within the liver parenchyma (7). Cirrhosis leads to decreased production of the anticoagulant proteins C and S (8). Cirrhotic patients also demonstrate chronic nutritional deficiency leading to low albumin levels. Low serum albumin is associated with an increased risk of thrombosis (9). Another example is patients with nonalcoholic steatohepatitis (NASH), this condition leads to additional risk factors for thrombosis. NASH is tightly correlated with obesity. It is associated with increased levels of plasminogen activator inhibitor. Thus, patients who are transplanted for NASH may not only be at risk for microthrombotic complications in the liver because of an altered pro-thrombotic state, but could also be at increased risk for macrothrombotic complications from altered regulation of the coagulation cascade (10).

2.2 Donor factors

Donor-related risk factors contribute to the severity of IRI in liver transplantation. Liver steatosis, older donor age, prolonged ischemic time, nature of organ recovery are risk factors for increased hepatic IRI. Liver donors with these risk factors have been labeled as “marginal”. However, because of the high risk of mortality while awaiting a liver transplant, there is increased demand for using these "marginal"

organs (11). In the US the reality for many transplant centers is that those marginal donors are the only way to increase OLT and reduce the death rate for those waiting.

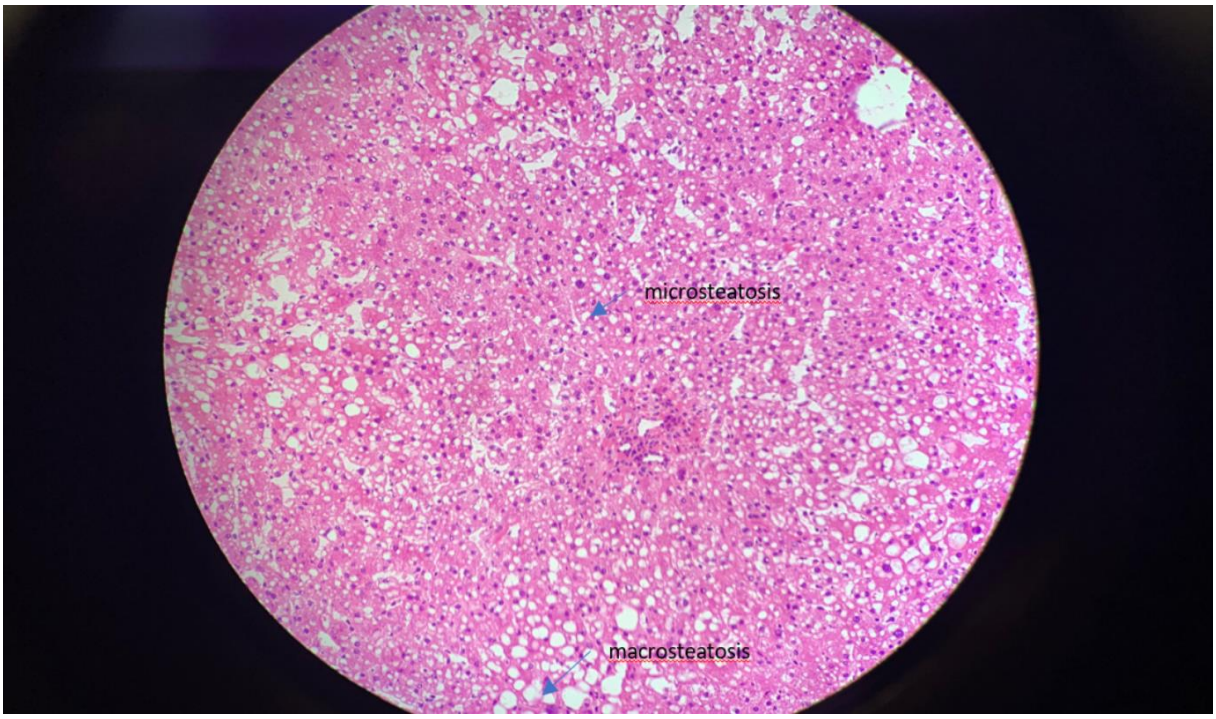
At IUPUI almost 40% of all liver grafts come from these marginal donors. That is why it is so important to understand the impact of IRI on them. Two particular and very common marginal donors are the fatty liver graft and DCD graft.

2.2.1 Steatosis in hepatic IRI

Hepatic steatosis is a common problem in Western countries, In the United States, the prevalence of fatty liver disease ranges from 10-46%. The major risk factors for fatty liver are: central obesity, type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome. Fatty liver disease can be subdivided into two major groups - Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). NAFLD is a condition in which excess fat is stored in liver. This buildup of fat is not caused by heavy alcohol use. When heavy alcohol use causes fat to build up in the liver, this condition is called alcoholic liver disease. NAFLD is also known as a simple fatty disease in which fat accumulates in liver with little or no inflammation or liver cell damage. Simple fatty liver typically does not progress to cause liver damage or complications. It is related with food intake. NASH is a form of NAFLD in which there is a presence of hepatitis—inflammation of the liver—and liver cell damage, in addition to the fat storage. Inflammation and liver cell damage can cause fibrosis, or scarring, of the liver. NASH progressively may lead to cirrhosis or liver cancer (12). Steatosis can be characterized quantitatively and qualitatively. The

quantitative evaluation is based on the percentage of hepatocytes containing cytoplasmic fat inclusions. There are three different degrees of fatty infiltration: Mild-up to one third of hepatocytes containing fatty inclusion; Moderate with inclusion between one third and two thirds; and Severe steatosis with two thirds or more of the hepatocytes containing fat. Currently, steatosis is usually considered as mild, moderate, or severe if, respectively, less than 30%, between 30 and 60%, or more than 60% of hepatocytes have fat vacuoles within the cytoplasm (13). Another important separation from the transplant stand point involves macrovesicular and microvesicular steatosis. In macrovesicular steatosis, hepatocytes contain one large vacuole of fat, which displaces the nuclei to the cell periphery (Fig.1). This type is most commonly associated with obesity, diabetes, or alcohol abuse. In microvesicular steatosis, the cytoplasm contains many small fatty inclusions and the nuclei remain in the center of the cell. This type of steatosis is caused by dysfunction of the mitochondrial oxidation, which is usually related to metabolic disorders. The impact of micro- versus macrovesicular steatosis on liver injury remains controversial, but it is widely agreed that the macrovesicular steatosis is worse than micro one.

FIG 1. THE DIFFERENCE BETWEEN MACRO AND MICRO STEATOSIS IN HEALTHY 38YO LIVER DONOR



What are the clinical results in using steatotic livers? For the first time Portman in 1987 suggested an association between severe donor steatosis (>60%) and primary nonfunction (PNF - defined as the patient's death or the need for retransplantation.) after transplantation (14). Currently, about 3 to 6% of patients experience primary graft nonfunction, another 15 to 30 % of patients undergoing OLT experience temporary graft dysfunction (15). Primary graft non and dysfunction are life-threatening complications, which are associated with increased morbidity and mortality rates and increased cost due to a prolonged intensive care unit (ICU) hospital stay. Currently livers with macrovesicular steatosis > 60 % are not used due to high patient and graft lost (16, 17). Livers with moderate macrovesicular steatosis are used with caution and livers with mild steatosis are used routinely due to the same outcomes as non steatotic livers (18).

What is the mechanism of injury in the steatotic livers? The damage provoked by IRI is multifactorial. The impairment of the microcirculation is considered a major event of reperfusion injury in steatotic livers. The fatty accumulation in the cytoplasm of hepatocytes of a steatotic liver is associated with an increase in cell volume that reduces the size of the hepatic sinusoid space by almost 50% compared with a normal liver and will result in partial or complete obstruction of the hepatic sinusoid space (19, 20). This reduction in sinusoidal perfusion appears to arise initially from the effects of enlarged hepatic parenchymal cells, swollen with accumulated lipid, which widen the parenchymal cell plates and narrow and distort the lumens of sinusoids. It has shown that as a result of the structural alterations around them, the sinusoids become inefficient conduits of blood with resulting impairment of tissue perfusion, evidenced by the significant reductions in the numbers of perfused sinusoids per microscopic field (21). Another feature unique for steatotic livers is that they are much more susceptible than nonsteatotic livers to lipid peroxidation because of either their lower antioxidant defenses or their greater production of ROS (22). Mitochondrial ROS generation dramatically increases during reperfusion and mitochondrial structures are exposed to the attack of the ROS generated both outside and inside these organelles eventually leading to the dysfunction of important mitochondrial processes including those responsible for the ATP synthesis. It is well-known that steatotic livers synthesize less ATP than non-steatotic livers during post-ischemic reperfusion (23). Fatty degeneration induces a series of ultra-structural and biochemical alterations in hepatocyte's mitochondria. This lower ATP and adenine nucleotide content observed in steatotic livers could be caused by severe mitochondrial damage.

Steatosis leads to an accumulation of nonesterified fatty acids. Those fatty acids have an inhibitory effect on the oxidation system resulting in a decrease of acetyl-coenzyme A (CoA) production. Acetyl-CoA is an important precursor for the Krebs cycle and gluconeogenesis. Therefore, important energy sources of the hepatocytes, oxidation and gluconeogenesis are decreased in steatotic livers. During liver transplantation, the blood supply to the liver is interrupted and the liver parenchyma is exposed to significant ischemia, depleting the intracellular energy levels. Several other factors, including poor recovery after ATP depletion, appear to contribute to bile duct cell duct system damage after liver transplantation. In steatotic liver graft undergoing 6 h of cold ischemia, necrosis was the predominant cell death whereas no apoptosis signs were found (24). At last fatty livers have a dysfunction of the apoptotic pathway (25). Fatty hepatocytes have an increased degree and accelerated development of apoptosis.

2.2.2 Impact of brain death on ischemia reperfusion injury in liver transplantation

IRI is a constant companion of organ transplantation. For the first time the influence of brain death on IRI was described by Wilhelm et al (26). Brain death plays a significant role in IRI organ damage, being a catastrophic event, resulting in physiological impairment with major body hemodynamic imbalance (27). This event brings hypothermia, hypotension and electrolyte imbalance. This is followed by an activation of the sympathetic pathway with its contrary effects as high plasma levels of catecholamines causing further organ damage. This circulatory chaos leads to significantly reduced organ perfusion, affecting organ quality and causing severe

graft ischemia before procurement (28). Apart from the physiological and hemodynamic changes in brain dead donors, the role of immunological response of brain death seems crucial. The brain death is associated with major alternations on the upregulation and downregulation of proinflammatory and anti-inflammatory cytokines (29). It has been shown that those inflammatory pathways are contributing to an increased leukocyte infiltration into the graft and lead to structural and functional changes of the graft resulting in higher rates of acute rejection and primary nonfunction, lower graft quality, ultimately influencing short-term and long-term graft survival (30). For the first time Weiss at all. (31) has shown the major differences in terms of cytokine/chemokine production in brain death and living donors. In this study plasma concentration of soluble cytokines in brain dead donors such as IL-6, IL-10, TNF-a, TNF-b excelled those in living donors. There is a strong relationship between brain death-induced cytokine storm and IRI on poor organ function, further leading to complications such as PNF and early rejections rates.

There is another ongoing discussion about potential associations of the duration of brain death and organ quality after procurement (31). There are various publications (32, 33, 34) on this topic which are very controversial. The first group described the effects of brain death duration on delayed graft function, acute rejection and short-term and long-term graft survival, observing a decreased risk for all mentioned factors as a result of increased brain death duration. Based on their observation the authors assumed that the rationale for these findings might be the result of protective effects of appropriate donor resuscitation and ICU management, and the induction of organ protective factors. On the opposite side,

other publications clearly reported that prolonged brain death duration is an independent risk factor for graft damage (35, 36). There it has been shown that the cytokine production and cell infiltration in the donor graft increased continuously with duration of brain death time, thus, decreasing the liver graft survival.

In conclusion, the central nervous system plays a fundamental role in the regulation of molecular markers triggering inflammation and tissue damage, and brain death results in a breakdown of these mechanisms ('autonomic storm'), hence initiating the cascade of IRI in liver transplants.

2.2.3 *Ischemic reperfusion injury in donor after cardiac death liver graft*

The pathophysiology of cardiac death is markedly different from that of brain death. As compared to livers obtained from brain dead donors (DBD), in which there is no consistent proceeding cardiac arrest, DCD livers are subjected to additional hypoxic insult. One of the biggest differences between DCD and DBD is the donor warm ischemia, which involve continued energy consumption by living cells at body temperature. Lack of tissue perfusion with adequately oxygenated blood results in anaerobic metabolism, owing to rapid depletion of oxidative energy. Thus, the energy exhaustion leads to permanent cellular damage (37). Although energy depletion contributes to the increased incidence of primary non-function in DCD grafts, several other mechanisms of cellular injury have also been discovered. In terms of energy DCD livers are exposed to severe ischemia, which lack in DBD livers. This donor warm ischemia time is calculated from the point at which systolic blood pressure decreased below 50 mmHg or desaturation measured

by pulse oximetry fell to 80 percent (whichever came first) to the time of institution of cold perfusion in the donor. In this period all hepatic cells get depleted from their primary energy source – the pyruvate. In an anaerobic environment it is the primary substrate for ATP. Anaerobic metabolism exhausts intracellular pyruvate levels and the resulting ATP generation is considerably lower per molecule of pyruvate metabolized. The end product of this anaerobic metabolism is the generation of lactic acid. DCD liver allografts have an increase in lactate/pyruvate ratio (38) which has been attributed to the severity of ischemic injury (39). Higher lactate levels can be explained by lack of tissue perfusion following withdrawal of life support in DCD donors. In addition, hypotension and reduced oxygen saturation both contribute to lack of oxygen delivery in the tissues. This, in turn, leads to slowing down of oxidative metabolism within the mitochondria and an increase in anaerobic metabolic pathways to generate ATP for the maintenance of cellular integrity (40). The donor core temperature is maintained at room temperature near the “agonal phase”, so cellular metabolism proceeded at the usual rate until the organs had been cooled. The basic metabolism within cellular systems is significantly reduced, but not completely halted by hypothermia. Each 10◦C degree drop in the environment is likely to reduce the metabolism by 1.5–2.0 fold, so liver grafts stored at 0–4◦C before implantation are likely to retain up to 10 percent of basic metabolism, energy for which is driven primarily by anaerobic metabolism. On reperfusion of the liver graft in the new recipient, a reversal of energy balance occurs, with rapid restoration of pyruvate through aerobic glycolysis to enter the tricarboxylic acid cycle for oxidative metabolism. Until this occurs, anaerobic

metabolism continues with production of lactate, which is primarily responsible for tissue acidosis (41).

Another major difference between DBD and DCD livers in settings of IRI is ischemic cholangiopathy (IC). IC is defined as diffuse nonanastomotic biliary strictures, with or without prestenotic dilatations, in the presence of a patent hepatic artery (42). In general, rates of IC are approximately 3% for DBD and 16% following DCD liver transplantation (43). The development of IC is associated with significantly increased patient morbidity due to the need for multiple biliary procedures and repeat hospitalizations. Up to 65% of patients with IC require retransplantation or die (44, 45). This is actually the main hurdle using DCD livers and the major factor explaining their low usage. The development of IRI has been implicated in IC pathogenesis; consequences of impaired biliary epithelial regeneration and cytotoxic injury has been associated with this disease process. This particular injury in DCD liver can be explained by the fact that cholangiocytes are more susceptible to ischemic injury than hepatocytes and rapidly die upon reperfusion due to production of oxygen free radicals and low levels of endogenous antioxidants. On top of this, hepatocytes excrete conjugated bile acids (bile salts) into bile canaliculi. In their protonated form, bile salts are hydrophobic and toxic toward canalicular membranes (46). In vivo, hydrophobic bile salts are combined with phospholipids thus, neutralizing their detergent properties. Following transplantation, however, phospholipid secretion lags behind that of bile salts, and high hydrophobic bile salt/phospholipid ratios leads to biliary injury. Another source of injury during IRI is related with cholangio's progenitor cells. These cells are recruited from the

peribiliary glands. Ischemic injury to these glands, however, may result in impaired recruitment of progenitor cells, thereby leading to poor regeneration and IC (47). In conclusion, the usage of DCD livers is still very limited due to unfavorable outcomes in respect of DBD livers. This can be explained with the additional damage that they receive during IRI phase.

2.3 *Impact of Ischemic reperfusion injury on other organs after liver transplantation.*

Liver dysfunction and failure are serious and potentially fatal postoperative complications, related with IRI. Although the liver is the primary injured organ and liver dysfunction is widely recognized as a consequence of hepatic reperfusion injury, many other remote organs seem to be influenced during this process as well.

2.3.1 *Hepatic IRI associated with kidney injury*

Acute kidney injury (AKI) is a frequent complication after liver transplantation. The reported incidences go in up to 94% in recipients of deceased donor livers (48). The development

of AKI after OLT, even mild or transient, increases morbidity, mortality, and the risk of

chronic kidney disease (CKD) in the future (49). The cirrhotic patient has unique physiology – portal hypertension which induces splanchnic vasodilation with subsequent intrarenal vasoconstriction (50). Splanchnic vasodilation leads to hypotension which in turn leads to activation of the renin-angiotensin system. Upregulation of renin-angiotensin system cause severe reduction of glomerular filtration rate, urinary sodium excretion, and free water excretion. For majority of

cirrhotic patients this is a base line, which can get only worse during the liver transplantation and potentially transform in AKI due to IRI. In fact, this preexisting renal dysfunction is a well-known risk factor for AKI (51). Other mechanisms also play an important role in the pathogenesis of renal dysfunction after liver ischemic reperfusion injury. One of the most important process is the systemic inflammatory response, which can induce renal injury (52). Circulating levels of proinflammatory cytokines and transcription factors, just like IL-6, TNF-α, and HMGB 1, are increased and their release from the liver promote inflammatory changes in the kidney after liver IRI (53). The release of different cyto and chemokines during IRI is known as systemic inflammatory syndrome. These proinflammatory substances can upregulate endothelial adhesion molecules in distant organs such as the kidneys. Upregulation of renal endothelial adhesion molecules promotes leukocyte recruitment and extravasations to the renal interstitial space (54) with substantial tubular damage. Activated neutrophils during IRI aggregate in the subendothelial tubular space, where they release reactive oxygen species, enzymes, and cytokines, causing direct renal injury (55) and the recruitment of monocytes and macrophages leading to further aggravation of the oxidative injury. Another mechanism for AKI after OLT is damage of the actin cytoskeleton in nephrocytes. It has been observed that loss of actin cytoskeleton may contribute to the development of renal tubular and endothelial apoptosis (56).

Another crucial factor contributing to AKI is the hypotension established after liver reperfusion and the subsequent usage of vasopressors. This is known as postreperfusion syndrome (PRS). PRS is characterized by a decrease in systemic vascular resistance, hypotension, impaired cardiac output, and an increased

pulmonary vascular resistance directly after reperfusion (57). This hemodynamic phenomenon has an incidence between 20% and 55% and is associated with higher in-hospital mortality (58). The damage occurred to the kidney during this period is driven by massive vasoconstriction in the afferent arterioles and subsequent ischemia of kidney glomerulus.

In conclusion, acute kidney injury after liver transplantation is multifactorial of origin, where the principal role is driven by reperfusion hypotension and general inflammatory response. AKI is a significant complication after liver transplantation as even mild postoperative AKI has a substantial impact on recipient outcomes including survival.

2.3.1 Hepatic IRI associated with lung injury

Along with kidney, lungs are one of the most common organs impact by IRI. Once again, cyto and chemokines released during liver reperfusion can provoke severe lung damage. Central role plays TNF-α, released from reperfused Kupffer cells, which interacts with pulmonary capillaries and elicits the expression of adhesion molecules, such as ICAM-1, leading to migration of neutrophils and subsequent lung injury (59). Along with TNF-α, a variety of different proinflammatory molecules, such as PAF, cytokine-induced neutrophil-chemoattractant protein, IL-6, and IL-18, as well as substance-P (60), are released from the reperfused liver and have been found to mediate lung injury after hepatic ischemia reperfusion. Another important part of the lung injury comes from local produced cytokines - such as TNF-α from alveolar macrophages and macrophage

inflammatory protein (MIP)-2 (61). The importance of the locally sustained pulmonary inflammation is in the fact that local produced cytokines can act longer than the original hepatic cytokines. Thus, the effect can persist for days. Those local cytokines plays a role of “shrapnel” after the initial reperfusion explosion. Another important mechanism of pulmonary damage is the translocation of endotoxins into the systemic circulation. Bacterial translocation occurs after OLT under vascular control even after the creation of a portosystemic shunt (73). In particular, insufficiency of Kupffer cells during reperfusion allows the spill-over of endotoxin in the pulmonary capillaries, stimulating TNF-α, IL-6, and MIP-2 production and subsequent neutrophil infiltration of the lungs (74).

Finally, oxidative stress during hepatic IRI is another factor to play a crucial role in the development of lung injury. Although the lung resists oxidative stress much better than every other organ, still ROS can damage the alveolar endothelium.

Another source of lung injury comes through liver transplant surgery. During the hepatectomy phase and especially reperfusion blood pressure is running low. This justifies the usage of vaso pressors and substantial amount of intravenous fluids- which easily can leak in lungs (third space) and provoke lung damage such as pulmonary edema.

In conclusion: Lung injury and acute respiratory distress syndrome after can severely complicate the postoperative course following liver transplantation. This damage is driven by cytokines, ROS, intestinal endotoxins and fluid resuscitation efforts. It is an important source of morbidity and mortality.

2.3.2 Hepatic IRI associated with intestine injury

The intestine is the biggest organ in human body and is tightly related with the liver during IRI. The damage done on intestine comes in different forms of intestinal dysfunction, including motility, transit time, and absorption function changes (63). The intestine injury comes in different forms. First, there is remote intestinal mucosa oxidative injury that results from the effect of liver-produced ROS, which are released in the systemic circulation. These oxidative radicals can damage the tight junctions between enterocytes, which lead to increased permeability and gut barrier failure (64). Another important mechanism is congestion of the portal venous system during the hepatectomy phase. During this phase the portal vein is clamped with arterial flow completely patent. Thus, the blood comes to the intestine and pancreas but can't leave them since the portal vein is clamped. This leads to intestinal congestion. Bowel wall edema can progress to intra-abdominal hypertension resulting in further compromise of intestinal perfusion and gut barrier dysfunction (65). Another aspect is the decreased bile production and intestinal motility after OLT. Bile has a trophic role for the intestinal mucosa. It binds to intraluminal endotoxin and bacteria creating nonabsorbable complexes and contains secretory IgA, which has antibacterial properties. The decreased intestinal motility results in increased intestinal bacterial load, which in turn has been shown to cause intestinal mucosal injury (66). Another way to damage the intestine is through apoptosis. It is implicated in gut mucosal injury following liver ischemia and reperfusion, since it is already known that extracellular free radicals can induce cell apoptosis. It has been reported that oxidative stress induces damage in enterocyte cell membranes and DNA, activating apoptotic pathways and thus disrupting the

mucosal barrier (67). During this process, enterocytes create sub epithelial crypts and “blubs”—microscopically known as Gruenhagen’s space (68). Alterations in the expression of adhesion molecules between the cellular membrane and the matrix in the villi is responsible for apoptotic intestine cell death. Lastly there is remote intestinal injury which is a consequence of venous congestion of the gut during the hepatectomy phase. Here we have increased intestinal permeability, endotoxinemia, and morphologic changes in the intestinal mucosa which can persist for days.

In conclusion, during OLT the intestine can receive major injury due to the nature of the surgery. In certain cases bowel ischemia can evolve to severe sepsis or mesenterial thromboses, which usually are fatal.

2.3.3 Hepatic IRI associated with pancreas injury

The etiology of pancreatic dysfunction after OLT has not yet been clarified. However, among the risk factors proposed to interpret hyperamylasemia following liver transplantation are chronic liver disease and portal congestion caused by the vascular control of the liver during liver transplant procedure. The key factor of post OLT pancreatitis is thought to be the production of ROS, resulting in remote organ injury. After reperfusion it was found in the portal blood high content of MDA, amylase and c-peptide levels which is direct evidence of pancreatic necrosis (69). Same pancreatic damage can be obtained during extended liver resections, where Pringle maneuver (vascular clamping of the liver pedicle) is used. Different studies have been done in this setting with similar conclusions - increased incidence of

postoperative hyperamylasemia and that the severity of hyperamylasemia is increased as the vascular occlusion time is prolonged (70, 71).

In conclusion - pancreatic injury, in the form of acute pancreatitis, is a rare but severe complication of liver transplantation. Although multiple factors have been implicated, it seems that remote oxidative burst plays a pivotal role.

2.3.4 Hepatic IRI associated with heart injury

The heart is the only organ where even small changes in vitality can bring potential death and severe morbidity. In the settings of OLT the heart starts to become damaged during as a result of cirrhosis. A more compromised liver function correlates with hyperdynamic circulation, cirrhotic cardiomyopathy, renal dysfunction with alterations in serum electrolytes and impaired coagulation resulting in a higher need for blood product transfusions. Of these, cirrhotic cardiomyopathy is the single and most relevant factor in the cardiac assessment before OLT. It is characterized by an increased stiffness of the cardiac fibers associated with hypertrophy, fibrosis and subendothelial edema. This configures a systolic and diastolic dysfunction, electrophysiological abnormalities and chronotropic incompetence. As with every organ, the damage comes with the excessive amount of cyto and chemokines. It has been demonstrated a significant correlation between the concentration of TNF-a obtained from the flushed blood at the end of the irrigation of the liver graft, and the need for vasopressors to treat hemodynamic instability after reperfusion (75). Another major factor involved in the reaction to graft reperfusion is the complement system. Activation of certain

elements of the complement system (such as C3 and C5 proteins) is related to hemodynamic derangements of the post reperfusion period (76) and subsequent post reperfusion syndrome (PRS). This syndrome is the initial and most important clinical heart response after liver reperfusion. It is defined as decrease in mean arterial pressure greater than 30% below the baseline value, lasting for at least 1 min, occurring during the first 5 min after reperfusion of the liver graft (unclamping of hepatic hilum). The incidence is high and goes up to 77% of all OLT (77). As stated before, PRS is the initial response of the heart to reperfusion. It is also an excellent prognostic for other short- and long-term cardiac issues such as – atrial fibrillation, stress cardiomyopathy, sudden cardiac death etc.

In conclusion – Cardiac changes after reperfusion starts with PRS and can evolve to cessation of the heart. Those changes can and should be anticipated in order to prevent any cardiac rhythm changes which are an important source of morbidity and mortality after OLT.

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Chapter III:

Indiana University Purdue University experience – OrganOx trial and laboratory data

1 OrganOx trial

1.1 Background and rationale on usage of normo-perfusion machine

Liver transplantation is the only effective treatment for many patients with liver disease. For patients with liver failure, techniques for supporting liver function provide only limited and temporary benefit as a bridge to transplantation. In the US, cirrhosis is the most common indication for liver transplantation (42%), other indications include cancer and acute hepatic necrosis. Over the last two decades, liver transplantation has become a victim of its own success: many more patients are referred for transplantation, but the number of suitable grafts from deceased organ donors has increased more slowly. The donor organ shortage constitutes a serious risk for patients with liver failure. It is the principal cause of increasing waiting lists and the death of patients on the waiting list worldwide. In 2016, 7,256 patients underwent liver transplantation in the USA, but 10,143 patients were added to the waiting list, and there was a mortality rate of up to 19% on the waiting list. This shortfall is typical of liver transplantation services around the world. Great efforts have been made in recent years to increase the referral of organ donors, but an increasing proportion of deceased donors are suboptimal. These include donors declared dead by cardiovascular criteria- DCD and other 'extended criteria' organ donors (such as older age, steatosis, etc.). There has been a much smaller increase in the number of standard criteria ('ideal') organ donors.

Much emphasis is now placed on optimizing the condition of those organs that are available, to enable an increased number of higher risk organs to be transplanted safely. The use of a higher risk organ does constitute a greater risk to the recipient, with a higher probability that the organ will never function and require immediate replacement (primary non-function; PNF), that it will function poorly and place the patient at risk of other complications (early allograft dysfunction; EAD) or that it will lead to later complications - multiple stricturing of the biliary tree (ischemic cholangiopathy; IC). The serious effects of the organ shortage resulting in many patients dying on the waiting list, has led to increased interest in using donor livers which were formerly thought unsuitable for transplantation. The use of these ´extended criteria donor´ (ECD; also called 'marginal' or 'high risk') livers for liver transplantation is now seen as essential if liver transplant centers are to address the demand. Several donor parameters have been identified as relative risk factors for poor outcome, including age; steatosis; DCD donation; split livers; prolonged cold ischemia time (>12 hours) etc.

A further strategy in the quest to use higher risk donor organs successfully is that of 'reconditioning' after retrieval – using techniques to reverse the injury sustained by the organ before and during the process of retrieval and treating the organ in such a way as to minimize the immediate damage that occurs after transplantation (ischemic reperfusion injury). Treatment of the organ during preservation has major logistic and ethical advantages over any attempt to achieve the same effects by treating the donor (in the US and many other countries therapeutic interventions before declaration of death that are of no potential benefit to the donor are not permitted).

At the moment the flow of oxygenated blood ceases, the supply of oxygen, cofactors, and nutrients stops along with the means of disposal of metabolic waste products. Anaerobic metabolism continues (at a temperature-dependent rate), leading to depletion of energy stores, mainly adenosine tri-phosphate (ATP), with a concomitant build-up of an acidotic milieu. ATP is required for energy-dependent cellular functions. The breakdown of ATP during ischemia also generates substrates for the production of reactive oxygen intermediates on reperfusion and initiates the cascade of ischemic injury. Prevention of ATP depletion is therefore an important target of innovative preservation methods. It has been shown that providing an oxygen supply to the organ can prevent ATP depletion and preserve viability of the liver.

Organs retrieved for transplantation undergo injury at several consecutive stages: 1) warm ischemia prior to preservation, 2) cold preservation injury, 3) ischemic rewarming during surgical implantation and 4) reperfusion injury. These consecutive events lead to a cumulative cellular injury that may not be compatible with recovery after transplantation. Standard clinical practice involves flushing and cooling the liver in situ with preservation solution. Typically several liters of cold preservation fluid are used both in situ and after removing the organ from the donor and before packing for transport and storage. Additional cooling may be provided by topical frozen saline slush both in situ and ex situ. After retrieval, the organ is placed in sterile plastic bags for transportation and stored in an ice-box in preservation solution until transplantation. Although the available preservation solutions differ in chemical composition, the function is essentially the same: to prevent cellular swelling

and death caused by fluid shifts as the membrane ion-exchange pumps cease operating in the cold environment. Although cold preservation slows metabolism by 1.5- to 2-fold for every 10°C drop in temperature, considerable metabolic activity still occurs at 1°C. This leads to accumulation of metabolic products which act as substrates for metabolism that takes place when the organ is re-perfused with oxygenated blood – the basis of the ischemia-reperfusion phenomenon. Cold storage causes injury to the graft regardless of other factors. It has been known for many years that extended preservation time with cold preservation solution has a deleterious effect on organ viability with a clear correlation between cold-ischemia time and post-operative primary graft function. Preservation injury is a more critical issue in higher risk donor organs. Good quality livers can tolerate preservation periods even up to 18 hours, while higher risk grafts must be implanted much more quickly in order to reduce the risk of potentially fatal graft dysfunction.

Machine perfusion systems can be divided in two major groups – Hypothermic Machine Perfusion (HMP) and Normothermic Machine Perfusion (NMP). HMP of the kidney has been shown to significantly improve the preservation compared with static cold storage, in terms of immediate graft function and medium-term outcome. Now days almost every kidney transplant center in US uses HMP. Trials trying to evaluate the impact of HMP onto the liver graft are ongoing. Normothermic machine perfusion gives a more ideal physiological approach using oxygenated blood at normal body temperature. Warm perfusion has the added

advantage of allowing more effective viability assessment of the organs while on the circuit (using multiple perfusion dynamic and biochemical parameters).

Initial experience with HMP was started at King's College Hospital in 2012. 20 subjects underwent liver transplantation using donor organs preserved throughout by normothermic perfusion. After assessing the feasibility and logistics of the project, it was decided to extend it to a multinational phase two clinical trial.

1.2 *Primary and secondary objective of the OrganOx study*

TAB 1 PRIMARY AND SECONDARY OBJECTIVE OF THE ORGANOX STUDY

	OBJECTIVES	OUTCOME MEASURES/ENDPOINTS
PRIMARY	To compare the effect of NMP to SCS in preventing preservation-related graft injury	Severity of immediate graft injury as measured by early allograft dysfunction (EAD).
SECONDARY	To compare graft and subject survival between NMP and SCS livers.	Primary non-function rates: Irreversible graft dysfunction requiring emergency liver replacement during the first 10 days after liver transplantation. Graft survival rates at 30 days, 3 months, and 6 months following transplantation. Subject survival rates at 30 days, 3 months, and 6 months following transplantation.
	To compare evidence of postreperfusion syndrome between NMP and SCS livers on transplantation.	Assess mean arterial pressure (MAP) pre- and post-reperfusion and requirement for vasopressor use.
	To compare biochemical liver function between NMP and SCS livers.	Bilirubin, GGT, ALT, AST, ALP and INR at days 1-7, day 30, month 3, and month 6 post-transplant. Lactate at days 1-7 while the subject is in ICU.
	To compare evidence of ischemia-reperfusion injury between NMP and SCS livers.	Post-reperfusion biopsies will be compared to baseline pre-reperfusion biopsies and graded according to standard histological criteria.
	To compare evidence of biliary complications between NMP and SCS ivers.	Incidence of biliary investigations and/or interventions between 7 days and 6 months post-transplant.
	To assess the feasibility and safety of NMP as a method of organ storage and transportation.	Incidence of livers randomized but not transplanted and reasons for not transplanting.
	To compare organ utilization between NMP and SCS.	Incidence of one or more of the following per randomized liver: (i) EAD; (ii) discard (non-transplant) of aretrieved liver; (iii) primary non-function.
	To assess the health economic implications of normothermic liver perfusion.	Logistical and healthcare costs (length of stay in ICU and hospital) and quality of life measures.

The severity of immediate graft injury as measured by early allograft dysfunction (EAD). The study is powered to demonstrate a reduction in EAD from 25% to 10% in NMP versus static cold perfusion (SCS). EAD is a binary outcome defined by the presence of one of the following 3 outcomes:

1. Serum bilirubin ≥ 10 mg/dL at day 7 post-transplant
2. International normalized ratio ≥ 1.6 at day 7 post-transplant
3. ALT or AST > 2000 IU/L within the first 7 days post-transplant

DONOR INCLUSION CRITERIA

1. DBD donor aged 40 years or greater
2. DCD donor aged 16 years or greater
3. Liver allograft from donation after brain death (DBD) or donation after circulatory death (DCD) donors

RECIPIENT INCLUSION CRITERIA

1. Subject is 18 years of age or greater
2. Subject is registered as an active recipient on the UNOS waiting list for liver transplantation
3. Subject, or legally authorized representative, is able and willing to give informed consent and HIPAA authorization
4. Subject is able and willing to comply with all study requirements (in the opinion of the Investigator)

Donor livers have been randomly assigned to NMP or SCS with 1:1 allocation as per a computer-generated randomization schedule using variable block randomization using the following stratification factors: participating (recipient) center and by donor type (DBD or DCD).

1.3 Material and Methods

From September 2017 to January 2020, our center matched 24 patients as part of the global US OrganOx trial (WP01 US trial protocol). 10 patients received liver transplant with OrganOx normoperfusion machine and 14 patients without (standard cold ischemia preservation) as a control group. Primary and secondary objectives are reported in Tab. 1. Outcomes were compared 1:2 with matched control liver transplant recipients with conventional static cold storage (SCS) grafts (for additional six patients). Control participants were selected from a pool of 50 adult deceased donor liver transplants at Indiana University in Indianapolis over the previous 12 months based on closest matching for (1) recipient Model for End-Stage Liver Disease (MELD) score, (2) donor age, (3) type of procurements brain death vs donor after cardiac death.

All patients signed informed consent prior to the organ transplant. Randomization was obtained through protected server part of the trial. Livers allocated to the intervention group were preserved using the NMP system (Organ Ox Metra). Briefly, the OrganOx was calibrated and primed in OR once a suitable liver graft was deemed acceptable for transplantation. Due to flying restrictions, all organ procurements were completed locally within Indianapolis city hospitals. Machine

perfusate consisted of 500 mL of Gelofusine (B. Braun, Melsungen, Germany) and 3 U of type O packed red blood cells.

Sodium bicarbonate 30 mL 8.4% was added as needed to maintain pH between 7.35 and 7.45. A full description of the OrganOx metra perfusate composition is listed in the original OrganOx protocol (not showed here) .

All livers were procured in standard fashion, flushed in situ with histidine–tryptophan–ketoglutarate solution (HTK), prepared, and cannulated on a “back table” at the donor hospital. Prior to perfusion, all liver grafts were primed with 500 mL 5% human albumin prior to NMP, with the intention of washing out carryover intrahepatic HTK to the perfusion circuit.

Liver grafts were monitored during NMP with interval determination of blood gases (pH, lactate), alanine aminotransferase (ALT), AST, and total bilirubin at perfusion start and every 2 h thereafter. Blood glucose was manually entered every 4 h, and the Nutriflex infusion was automatically adjusted by the device accordingly. Liver perfusion quality was documented by variation in perfusate pH, lactate concentration, and perfusion vascular stability and by hourly bile production. Once recipient hepatectomy was completed, the NMP was discontinued. Thereafter, all liver grafts were further flushed with cold HTK solution immediately before being brought into the surgical field.

Surgical implantation techniques were identical between NMP and SCS groups. All livers were transplanted with standard piggy-back caval replacement, without bypass or temporary portocaval shunt.

Posttransplant care in both groups was performed following standard protocols including tacrolimus-based immunosuppression, as appropriate, and sirolimus (month after OLT) if preexisting renal dysfunction was present.

Statistical analysis:

Data is represented as medians and ranges and as means plus or minus standard deviations as necessary. The Mann–Whitney U test, and two-way analysis of variance with Bonferroni multiple comparisons were used to analyze differences between continuous variables. The Fisher exact test was used to compare proportions between groups for categorical outcomes. Overall comparisons between NMP and SCS groups was performed with a 95% confidence interval. A p-value <0.05 was considered significant.

1.4 IUPUI results in OrganOx trial

Recipients characteristics - In total, ten participants underwent transplantation with livers perfused by NMP. Transplant indications and preoperative MELD are shown in Table 1. Median recipient MELD was 21 (range 9–32) in the NMP group versus 20 (range 9–27) in the SCS group (p = 0.38). Median recipient age was 58 years (range 38–67 years) in the NMP group versus 59 years (range 47–68 years) in the SCS group (p = 0.28) (Table 2). The only significant variable between the two groups was the cold ischemia time, which was significantly higher (as expected) in the SCS group (Table 3).

Donor characteristics: In total, 10 liver grafts were procured, and were successfully perfused using OrganOx device and transplanted. Four grafts (40%) were from DCD donors (Maastricht

category III), and six (60%) were from DBD donors. The median donor age was 52 years (range 21-68 years) in the NMP group versus 51 years (range 23-62 years) in SCS controls (p = 0.93) (Tables 3).

Normothermic machine perfusion: A crucial part of the liver procurement was put on priming the OrganOx device appropriately. The machine perfusate is a complex mixture of blood, human albumin, glucose, and other nutrients. In all brain death organ procurements, the device was primed once liver was deemed as transplantable, thus minimizing cold ischemia. During DCD organ harvesting, that was done once liver was out.

Liver transaminases (AST, ALT) in the recirculating NMP ex vivo perfusate circuit rose progressively in all cases. Perfusate AST was notably higher for DCD compared with DBD livers.

All perfusions required supplemental sodium bicarbonate to maintain physiological pH, but all grafts cleared lactate rapidly while on circuit. All NMP grafts demonstrated stable portal vein and hepatic artery flow rates.

TAB 1. DONOR AND RECIPIENT CHARACTERISTICS IN ORGANOX GROUP

Patient	Donor age	Donor BMI	Type	CIT min	NMP time	Recipient Age	Indication for LT	MELD-Na	Complications	Length of H stay (day)
1	63	34	DBD	125	280	58	NASH	34	ERCP, CVVH	39
2	60	24	DBD	135	300	62	HCV HCC	15	none	7
3	21	26	DBD	140	280	59	ETOH	25	none	19
4	68	21	DCD	130	310	66	NASH	21	none	8
5	68	27	DBD	150	350	47	AUTOIMMUNE	23	AKI, ERCP	13
6	47	25	DCD	140	320	38	WILSON	6	ERCP	19
7	53	38	DCD	120	320	67	NASH	24	none	8
8	48	33	DBD	140	380	64	NASH	16	none	13
9	49	26	DCD	130	350	59	ETOH	29	none	12
10	63	31	DBD	130	380	61	NASH	27	none	8

TAB 2. DONOR AND RECIPIENT CHARACTERISTICS IN SCS GROUP

Patient n.	Donor age	Donor BMI	Type	CIT min	Recipient age	Indication for LT	MELD-Na	Complications	Length of H stay (day)
1	55	29	DBD	270	55	HCV	20	none	13
2	57	21	DCD	300	58	Crypto	18	none	14
3	46	30	DBD	350	62	NASH	26	none	23
4	42	29	DBD	330	69	NASH	24	none	7
5	62	34	DCD	300	59	NASH	19	none	8
6	23	23	DBD	380	57	NASH	14	GJ tube	46
7	27	29	DBD	420	63	NASH, HCC	16	none	7
8	52	36	DBD	330	66	NASH	27	none	19
9	60	33	DBD	300	55	HCV, HCC	19	none	31
10	50	19	DCD	280	70	NASH	24	none	7
11	54	28	DBD	330	66	NASH	22	ERCP	18
12	61	31	DCD	350	48	ETOH	21	none	9
13	47	26	DBD	290	47	ETOH	17	ERCP	23
14	52	30	DBD	310	39	AUTOIMMUNE	9	none	7
15	48	25	DBD	300	58	HCV	23	none	15
16	63	29	DCD	300	61	HBV, HCC	26	ERCP	17
17	52	31	DBD	310	55	ETOH	26	none	21
18	56	33	DBD	360	64	NASH	21	ERCP	8
19	61	29	DCD	380	55	HCV	25	none	22
20	42	30	DCD	290	67	HCV	27	none	23

DCD – Donation after circulatory death
DBD – Deceased brain death donor
NMP – Normothermic machine perfusion
BMI – Body mass index
LT – Liver transplantation
NASH – nonalcoholic steato hepatitis
HCV – Hepatitis C virus
ETOH – Alcoholic hepatitis
AKI – Acute kidney injury
ERCP – Endoscopic retrograde cholangio pancreatography
GJ – Gastro-jejunal tube.

TAB 3. SUMMARY OF DONOR AND GRAFT CHARACTERISTICS

	NMP (10)	SCS (20)	p-value
Donor age	52 (21-68)	51 (23-62)	0.93
Donor BMI	28 (21-38)	29 (19-34)	0.45
Cold ischemia time	134 (125-150)	320 (270-420) min	0.06
DCD/DBD proportion	4/10 (40%)	7/20 (35%)	0.53
MELD score	21 (9–32)	20 (9–27)	0.38

TAB 4. OUTCOME COMPARISONS BETWEEN NMP AND SCS CONTROL LIVER TRANSPLANT RECIPIENTS

Outcomes	OrganOx	SCS	p-value
30-day graft survival	10/10	30/30	-
6 mo graft survival	10/10	30/30	-
Recipient age, median (range)	58.1 (38 – 67)	59.1 (47 – 69)	0.31
Recipient MELD, median (range)	21 (9 – 32)	20 (9 – 27)	0.41
AST peak by day 5	1482 (96 – 3288)	1289 (279 – 3990)	0.51
INR day 5, median (range)	1.3 (0.9 – 1.4)	1.1 (1 – 1.5)	0.48
Bilirubin day 5, median (range)	3.3 (0.8 – 11.2)	3.1 (0.6 – 9.2)	0.52
Lactate 24h median (range)	1.4 (0.7 – 6.2)	1.3 (0.8 – 2.4)	0.75
Primary Non function	0/10 (0%)	0/20 (0%)	-
Early allograft dysfunction	3/10 (30%)	4/20 (20%)	0.47
Hospital stay	14.1 (7 – 39)	16.2 (7 – 31)	0.35
6 mo biliary complications	3/10 (30%)	4/20 (20%)	0.38

Post-transplant liver function assessment: There was no statistical difference between peak AST

levels within the first 5 days in OrganOx versus SCS group (p = 0.51). There was no difference

in bilirubin levels between groups on day 5 (p = 0.52). Comparison of coagulation parameters demonstrated stable, normal, uncorrected international normalized ratio (INR) synthetic function in both groups (p = 0.48). Arterial lactate was not significantly different between groups and normalized within 3 days (p = 0.75). Hospital stay was also similar between the two groups (p-0.35) Tab. 4.

Complications after liver transplant – All patients received US liver transplant doppler to assess the vascular patency. No arterial or venous (portal and hepatic vein) thrombosis was reported. In the NMP group we performed 3 ERCP on day 4/5 for rising total bilirubin. Two of the patients received biliary stent, which was removed after 2 months, the third patient was dilated without stent placement. From the SCS group four patients received ERCP, all with biliary stent placement. We did not observe any ischemic cholangiopathy in both groups. The assessment of EAD (early allograft dysfunction) was done using three criteria – 1) a bilirubin level > 10 mg/dL on day 7, 2) an international normalized ratio > 1.6 on day 7, and 3) an aspartate aminotransferase (AST) level > 2000 IU/L within the first 7 days after transplantation. In our series three (30%) patients had EAD because AST>2000UI. Of note, this resolved without any other graft consequences, in fact we did not observe any primary graft non function. In the cold storage group, we observed four patients (20%) again due to high AST.

1.5 *Discussion and Conclusions*:

First, limitations of our study include a small cohort size, limited matching between OrganOx and SCS groups, and relatively short (8 months) follow-up. As one of the top five busiest liver transplant programs in the US, we randomized a fairly low number of patients. There were several limiting reasons for this. OrganOx machine can be used only for local organ recovery since it is not approved for transport by air by FDA. Another limit of this device is that it needs a dedicated ground transport, since battery life is less than 30 min. This heavily limited the use of it to a few local hospitals. Another reason is the high complexity of the machine operation. All OrganOx cases have been done with a dedicated team of a perfusionist, OrganOx representative, transplant coordinator, research consultant and a special previously trained surgical team. Another unexpected event that put a stop for 8+ months of the whole program (globally in US) was an issue with the disposable tubing set. It was found out that the plastic tubes used to circuit the blood in the OrganOx machine could be easily contaminated. This led to a complete halt of the whole trial with subsequent changing of all disposable materials and resubmission to FDA for trial safety.

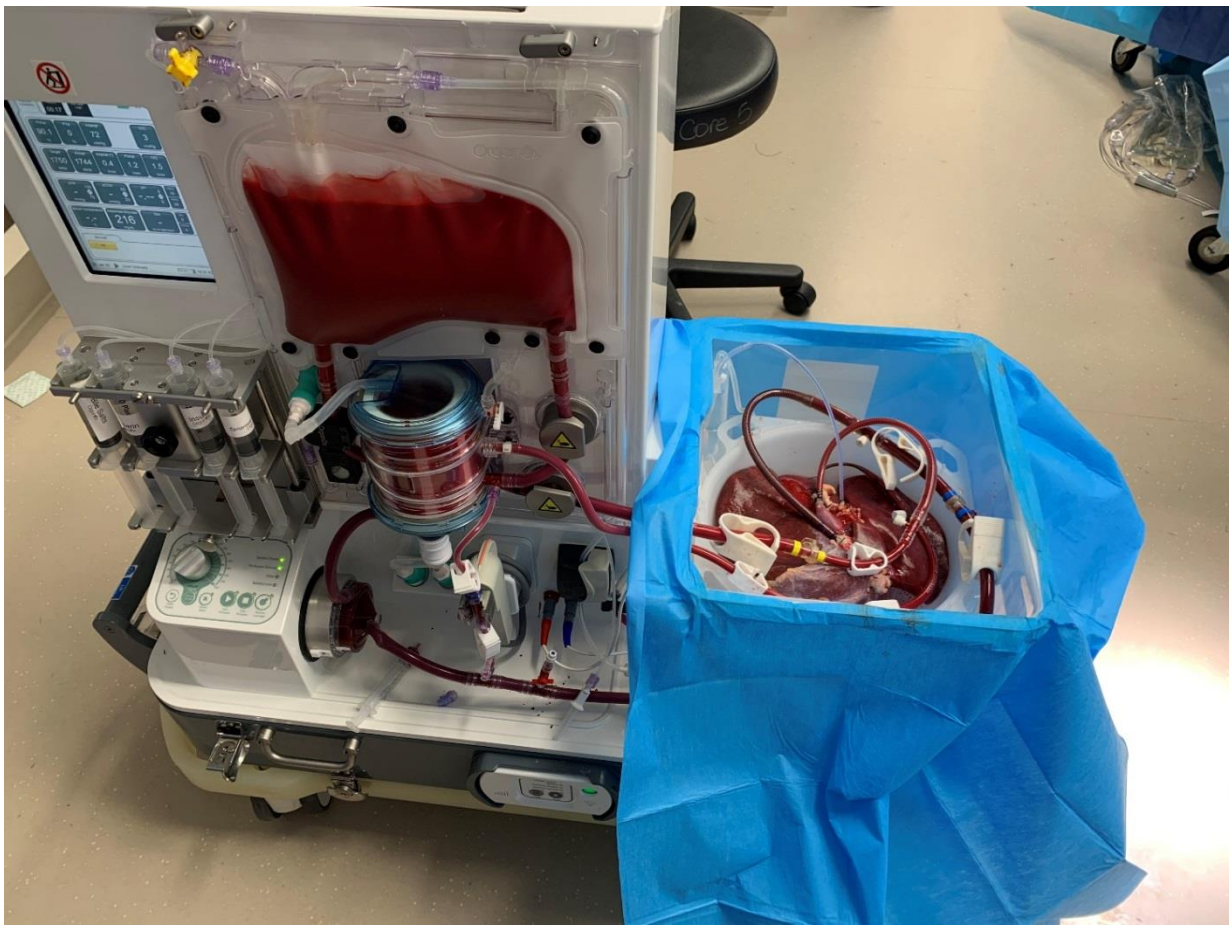
Nevertheless, despite all the hurdles we accomplished with success the OrganOx trial.

As far as responding to the primary and secondary objectives of the study, we concluded that the primary goal to evaluate the EAD in NMP group was done successfully. We had three patients with EAD due to high AST, with no early or late graft primary non function or other liver issues. It is important to note that all three-liver graft were from DCD procurement (with functional warm ischemia time

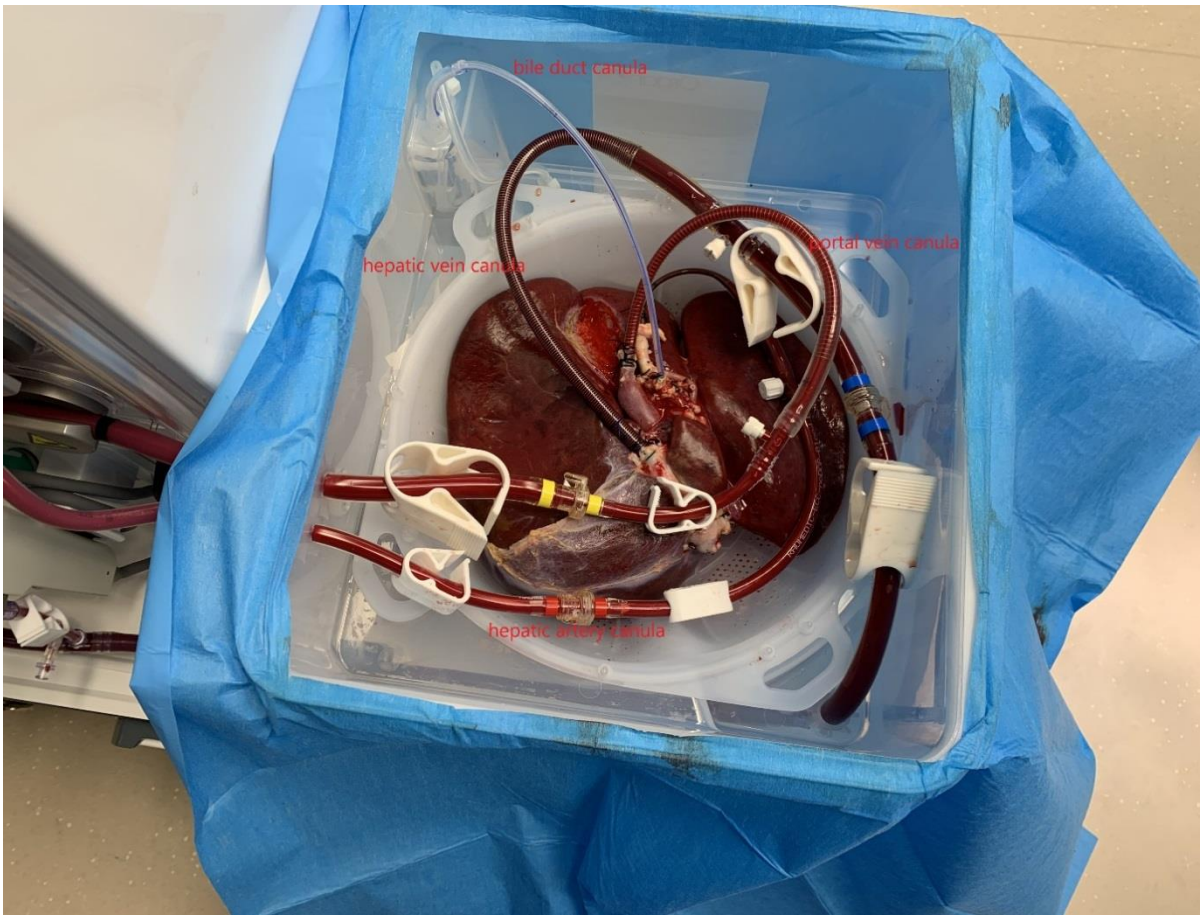
up to 30min) which can explain higher overall AST levels. Concerning secondary objectives, we did not find any primary non function in NMP group as well as 100% graft survival at 1 and 6 months. All biochemical markers were similar in both groups. Surprisingly, we reported three patients who required early ERCP. All procedures have been performed in the early post-operative period. All three patients have been followed once biliary stent has been removed and up to date (1 year), no ischemic cholangiopathy was noted. Despite the complexity of OrganOx, we deemed it as efficient, reliable and most importantly, safe to use.

This initial experience provides important groundwork for future trials that will explore the feasibility of this technology in expanding the currently limited donor pool.

PIC.1 OVERALL LOOK OF ORGANOX METRA MACHINE IN ACTION WITH LIVER INSIDE



PIC.2 CLOSE LOOK OF INCANULATION OF HARVESTED LIVER.



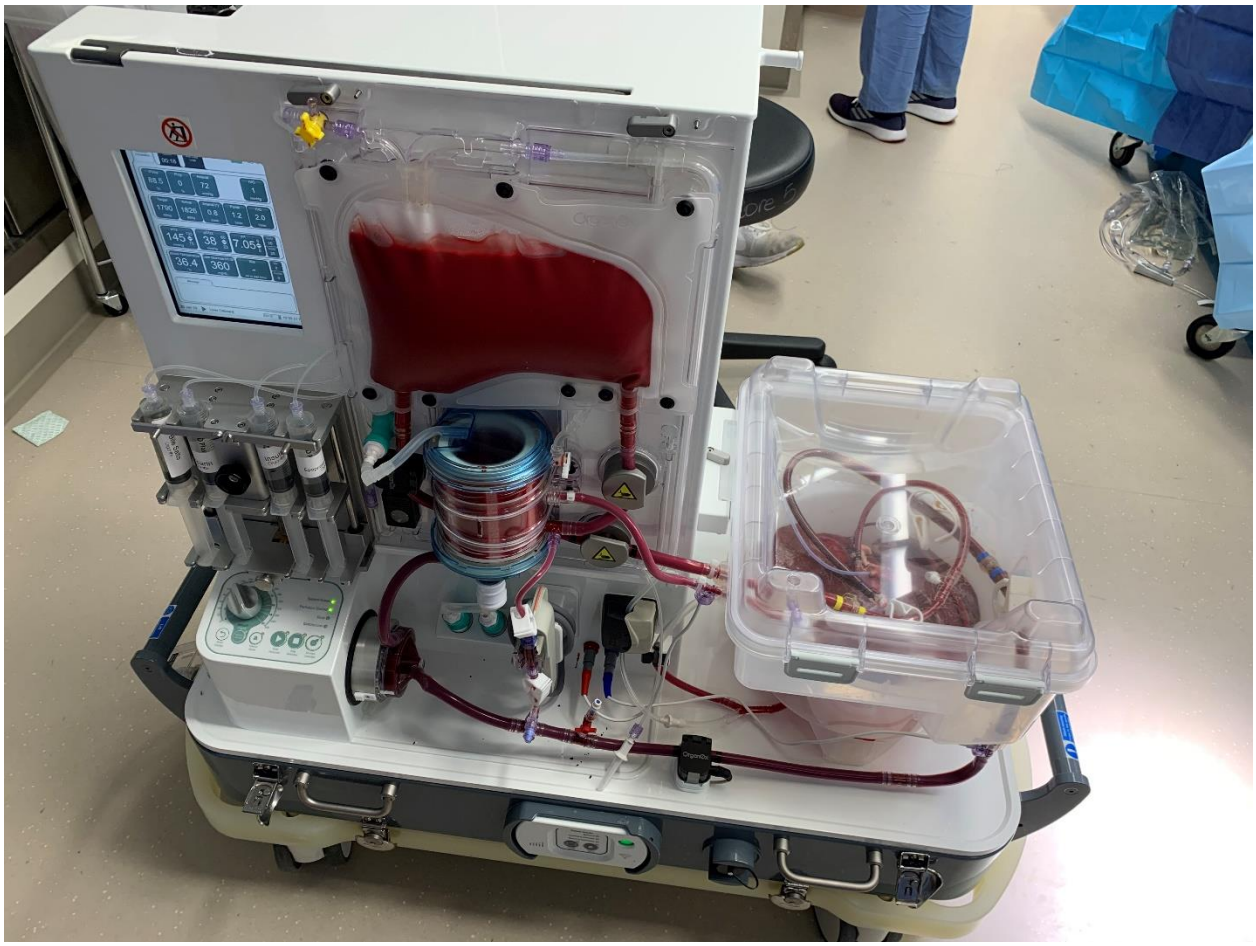
Pic.2 Close look of incanulation of harvested liver.

All cannulas and belonging vessels must be checked for kinking. Even a minimal vessel lumen irregularity can impair perfusion. Fortunately, this is detected by the machine initiating an alarm. Special attention needs to be made during the priming, since even a small amount of air bubbles can damage the organ. For this purpose, all incanulation is done under HTK preserving solution.

PIC.3 FRONT VIEW OF ORGANOX PERFUSION MACHINE

Pic.3 Front view of OrganOx perfusion machine. On the big LCD panel in real time are tracked different perfusion parameters such as, bicarb, glucose and pH. Arterial, portal and hepatic vein pressure is measured constantly providing optimal perfusion to the organ. Four different products are hooked up to the machine as an addition to the main perfusate – bile salts, heparin, insulin and epoprostinol (four syringes). The main bag contains three units of O group blood with additional nutritive perfusate (Gelofusine). This highly complex perfusion liquid aims to provide an ideal environment for the liver to recover after the cold ischemia during procurement. The liver function can be easily checked through serial blood draws and lactate clearing, pH assessment as well as bile production.

PIC.4 ORGANOX READY TO LEAVE OR



Pic.4 OrganOx ready to leave OR – a sterile container protects the liver inside and provides a good view of all tubes and vessels. Usually once liver is on pump, it will take between 30 and 60 min before it is safe to be packed and prepared for transportation. This is due to the fact in those 60 min, the liver needs to be closely observed and supply with bicarb in order to keep pH between 7.35-7.45. Failing to do so triggers an alarm. Once perfect homeostasis is achieved it is safe to move the organ.

2 Laboratory data

2.1 Cytokine and chemokine involvement in the setting of liver transplantation.

Cytokines and chemokines play an important role in liver transplantation as inflammatory mediators. “Cytokine” is a general term used for a diverse group of small soluble proteins (<20kDa) and peptides which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate direct interactions between cells and regulate processes taking place in the extracellular environment. They may lead to hemodynamic instability, graft damage, and alterations in different organs that cause multiorgan failure. The liver is home to a tightly regulated cytokine network. Hepatocytes are highly susceptible to cytokine activity in physiological and pathophysiological conditions, both acute and chronic. In the adult liver, approximately 30% of the liver’s cells are non-hepatocytes and include hepatic stellate cells, liver sinusoidal endothelial cells, macrophages (Kupffer cells), dendritic cells, and lymphocytes, which can produce a variety of cytokines, chemokines, and growth factors acting systemically on hepatocytes and nonparenchymal cells. Additionally, several cytokines are key mediators of the hepatic acute phase response. Reperfusion induces the activation of Kupffer cells and the loss of viability of endothelial cells, increasing the release of inflammatory substances, most important of which are cytokines. Graft reperfusion produces alterations of microvascular endothelial membranes resulting in increased permeability and, secondarily, damage to other organs such as heart and kidneys. Any of these cytokines might be induced upon acute liver injury; however, their involvement and kinetics in this process remain unclear.

Several clinical tests are routinely used to monitor liver dysfunction. These include increased elevated blood levels of the intracellular liver enzymes alanine transaminase (ALT) and arginine transaminase (AST), which are released upon hepatocellular damage. Total bilirubin is also used as a measure of liver function, as it indicates either impaired heme catabolism or cholestasis, a partial to complete blockage of bile flow. Finally, prothrombin time, reported as the international normalized ratio (INR), is a common blood clotting test used as a measure of liver biosynthetic function. However, all these tests suffer from poor sensitivity and specificity,

and it is uncertain how these tests relate to ischemia reperfusion injury (IRI), which is currently only identifiable by biopsy.

It has been more than half of a century since the first liver transplant. Now days this procedure is a standard of care without alternative for acute and end-stage liver disease. This procedure requires much organization, starting with the organ procurement and finishing with the long term follow up. Despite all these years of experience, there are a lot of unknown aspects of this complex operation. Ischemic reperfusion injury is a known complication which occurs physiologically in all organs without blood supplies. In the transplant field it is inevitable and is a source of severe morbidity and mortality. IRI is a multifactorial process starting with donor perfusion during organ procurement and culminated with reperfusion of the transplant organ. It is a complex process involving numerous cells, inflammatory substances, blood mediators etc. At present there is no treatment available to prevent hepatic IRI. The purpose of this study was to assess the association of preoperative and postoperative cytokine levels with the development of acute

kidney injury (AKI). Secondarily, to identify molecules involved in IRI and their relationship with damage to other organs.

3 Material and Methods

After approval by the institutional review board, written consent was obtained from 66 patients undergoing liver transplantation. This prospective study was done between January 2018 and June 2019 at Indiana university hospital. All patients received orthotropic liver transplantation. All combined and re-do liver transplants were excluded. We defined IRI in respect of AST level peak (first 48h after OLT) as follows: mild with AST<2000 U, moderate with AST<5000U and severe with AST > 5000UI. As per AKI we used the latest KDIGO group classification including all causes of acute renal dysfunction as indicated by an increase in serum Cr to 1.5-2 times from admission level within 7 days as mild. Serum Cr 2 to 3 times from admission level as moderate and serum Cr greater than 3 times than admission level as severe.

3.1 Cytokines, Blood Sample Drawing, and Processing

Blood samples were obtained pre-operatively on the day of the transplant (day 0) and at 4h, 8h after liver reperfusion, as well as day 1, day 2, day 7, day 14, day 30. From each blood sample serum was isolated and stored at -80 C until assessment by Luminex analysis.

Luminex assays - 38 Cytokines/Chemokines in sera were simultaneously measured using a magnetic bead-based multiplex kit (EMD Millipore, HCYTMAG-60K-PX38) according to the manufacture instruction. Analytes included cytokines: sCD40L, EGF, , FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, , IL-1α, IL-1β, , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, cytokine receptors - IL-1ra, chemokines - Eotaxin/CCL11, IFN-γ, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, TGF-α, pro-inflammatory cytokine - TNF-α and TNF-β, VEGF . The concentrations of analytes were calculated using Bio-Plex Manager v6.1 software (Bio-Rad, Hercules, CA).

4 Data Processing

Cytokine levels which were summarized as median values (25th–75th percentiles). A value of *P* _ .05 was considered significant. An alternative graphic representation of results was added. All calculations used SPSS 15.0 computer statistical package by SPSS, Inc (Chicago, IL).

5 Results

Patient characteristics – Table 1 shows donor age, gender, BMI, steatosis and type of donor (DBD/ DCD). As far as recipient characteristics we followed gender, age, MELD score prior OLT, BMI, primary diagnosis for OLT, cold ischemia and post reperfusion syndrome.

According to IRI and AKI status we divided all patients in two groups according to IRI – with ischemic reperfusion injury (IRI+) and without ischemic reperfusion injury (IRI-). Furthermore, we divided the main groups into two subgroups according to their AKI status. Tab 2 presents different cytokines involved in all four groups - IRI+AKI+; IRI+AKI-; IRI-AKI+ and IRI-AKI-. In Tab 3 same cytokines presented in standard value. We defined post reperfusion syndrome (PRS) as a usage of at least one vaso active pressor 3 hours after liver reperfusion, in order to keep blood pressure at 100/60 or higher. In the literature there is no consensus about the definition of PRS. Vastly it is accepted to define PRS as a decrease in mean arterial pressure (MAP) greater than 30% below the baseline value, lasting for at least 1 min, occurring during the first 5 min after reperfusion of the liver graft. In our center we are using a different approach in order to prevent PRS -five min prior of reperfusion, the anesthesia team start to give small boluses of Norepinephrine (or another vasoactive amine). Whenever clamped, the BP will stay relatively stable. Later on, if the patient experiences ischemic reperfusion injury, the BP will continue to decrease and continuous infusion of pressors will be required. It is important to clarify that, PRS not only is responsible for hypotension during graft reperfusion but also seems to have an impact on long-term postoperative outcomes. Hilmi et al. showed that patients with PRS had a lower 5-year survival rate than those without

PRS. It was also found that the days in the intensive care unit, the total number of hospital days, and the days on the ventilator were greater in patients with PRS. This is one of the reasons that the identification of factors contributing to the development of PRS has become the target of several investigations. In our cohort we found that PRS is tightly connected with ischemic reperfusion injury. In fact, 60% (20/33) of patients with IRI+ experienced severe post reperfusion syndrome and needed two or more vasoactive amins to keep blood pressure in standard parameters (p – 0.02). On the other side, in the IRI- group only 4 out of 28 patients required same amount of pressors.

We evaluated the liver quality in terms of macro steatosis. The liver steatosis was divided in two groups – above or below 20%. Livers with macro steatosis greater than 20% have been labeled as "marginal", because of the poor outcomes. In fact, 85% of livers with macro steatosis greater than 20% have been part of IRI+ group. This group had significantly higher liver steatosis (p – 0.18). Other donor characteristics such as age and BMI have been non statistically significant. Unexpectedly, the donor type – brain death or donor after circulatory death was also non statistically significant (p – 0.90). As far as recipient characteristics, we found that gender, age, BMI, MELD and primary diagnosis haven't been involved with IRI. Cold ischemia times were 28% longer in the IRI+ group than the control group (IRI-) achieving statistical significance (p – 0.03).

Several cytokines have been associated with the occurrence of ischemic reperfusion injury - G-SCF; IL-6; IP-10 and HSP90a. Not surprisingly IL-6, and G-SCF are in this group. There have been numerous publications of their involvement in early allograft dysfunction as well as in post-transplant inflammation and rejection. Rising

of their levels occurring right after the reperfusion and the peak was in less than 24 hours. This cytokine “storm” is also contributing to the severe hypotension and subsequent AKI. In fact, in the group IRI+AKI+ almost all patients have required 2 or more vaso active pressors post- transplant. Few anti-inflammatory cytokines such as IL-10, IL-1Ra and IL-4 were involved too. These levels have been marginally higher in the IRI- group.

As far as kidney damage assessment from IRI, we found that there was no long-term repercussion on the kidney function – one month Cr after OLT p – 0.274 and 12 months Cr post OLT p – 0.207.

TAB. 1 COMPARING DIFFERENT VARIABLES INVOLVED IN IRI DURING LIVER TRANSPLANTATION

Variable	**Level**	**IRI+AKI+**	**IRI+AKI-**	**IRI-AKI+**	**IRI-AKI-**	**P-value compares IRI+vs IRI-**
DONOR						
Age	NA	40(29/63)	40(26/66)	35(28/45)	36(16/63)	.248
BMI	NA	35(26/51)	32(22/53)	29(22/34)	27(19/52)	.242
Donor type						
	DBD	13(16)	12(17)	4(4)	27(30)	.090
	DCD	3(16)	5(17)	0(4)	3(30)	
Gender						
	Female	3(16)	5(17)	1(4)	9(30)	.325
	Male	10(16)	12(17)	3(4)	21(30)	
Steatosis						
	Greater 20%	9(16)	3(17)	0(4)	2(30)	.018
	Less 20%	7(16)	14(17)	4(4)	28(30)	
RECIPIENT						
Gender						
	Female	3(16)	4 (17)	2(4)	13 (28)	
	Male	13(16)	13(17)	2(4)	15(28)	
Age	NA	58(35/70)	48(21/65)	53(49/57)	56(37/72)	.450
MELD score	NA	21(16/34)	19(19/29)	25(20/31)	23(16/31)	.213
BMI	NA	29(19/38)	26(20/41)	27(23/32)	26(20/41)	.969
Primary diagnosis						
	Alcoholic	5(16)	4(17)	3(4)	12(28)	
	Autoimmune	3(16)	5(17)	0	3(28)	
	NASH	6(16)	5(17)	1(4)	9(28)	
	Other	2(16)	3(17)	0	4(28)	
Cold ischemic/min	NA	392(240/610)	350(230/540)	285(210/320)	294(220/510)	.003
PRS						
	2 pressors or >	14(16)	6(17)	0(4)	4(28)	.002
	1 pressor or none	2(16)	11(17)	4(4)	24(28)	

TAB. 2 DIFFERENT CYTO AND CHEMOKINES REPRESENTED IN MEAN VALUE BEFORE AND AFTER 24H POST LIVER TRANSPLANTATION

Cytokines	MDN value		Odds Ratio	95% CI	P - value
	Day 0	Day 1			
EGF	111.59	67.89	1.00	(0.99,1.00)	0.288
FGF2	81.57	67.37	1.00	(0.99,1.00)	0.167
Eotaxin	159.63	54.11	1.00	(1.00,1.00)	0.036
TGFa	6.59	13.21	1.00	(0.99,1.00)	0.137
GCSF	44.20	273.44	1.00	(0.99,1.00)	0.044
Flt3L	12.57	5.40	1.00	(0.99,1.00)	0.358
GMCSF	16.08	19.02	1.00	(0.99,1.00)	0.352
Fractalkine	84.04	110.00	1.00	(0.99,1.00)	0.197
IFNa2	26.45	31.29	1.00	(0.99,1.00)	0.329
IFNy	6.68	8.75	1.00	(0.99,1.00)	0.292
GRO	385.92	732.72	0.99	(0.99,1.00)	0.170
IL10	13.65	170.16	1.00	(0.99,1.00)	0.085
MCP3	61.14	20.61	1.00	(0.99,1.00)	0.305
IL12P40	28.36	21.23	1.00	(0.99,1.00)	0.621
MDC	883.37	564.32	1.00	(0.99,1.00)	0.174
HSP90a	19.23	164.26	1.00	(1.00,1.00)	0.001
IL13	12.76	8.15	1.00	(0.99,1.00)	0.462
IL15	6.91	14.71	1.00	(0.99,1.00)	0.313
sCD26	506.00	329.00	1.00	(0.99,1.00)	0.409
sCD40L	1951.20	1646.44	1.00	(1.00,1.00)	0.340
IL17A	5.85	10.50	1.00	(0.99,1.00)	0.158
IL1RA	35.15	644.91	1.00	(0.99,1.00)	0.048
IL1a	45.87	30.39	1.00	(0.99,1.00)	0.503
IL9	3.95	2.38	1.00	(0.99,1.00)	0.283
IL1B	2.83	2.17	1.00	(0.99,1.00)	0.694
IL2	3.83	10.37	1.00	(0.99,1.00)	0.257
IL3	0.70	0.70	1.00	(0.99,1.00)	0.265
IL4	7.84	4.50	1.00	(0.99,1.00)	0.283
IL5	3.59	5.20	1.00	(0.99,1.00)	0.417
IL6	21.06	124.47	1.00	(0.99,1.00)	0.097
IL7	5.13	7.50	1.00	(0.99,1.00)	0.383
IL8	111.79	220.12	1.00	(0.99,1.00)	0.759
IP10	525.20	1369.31	1.00	(0.99,1.00)	0.087
MCP1	429.83	1180.69	1.00	(1.00,1.00)	0.020
MIP1a	25.33	28.45	1.00	(0.99,1.00)	0.427
MIP1B	45.44	57.83	1.00	(0.99,1.00)	0.427
TNFa	15.81	58.17	1.00	(0.99,1.00)	0.063
TNFB	7.29	4.17	1.00	(0.99,1.00)	0.345
VEGF	161.93	91.03	1.00	(0.99,1.00)	0.235
IL12P70	5.41	6.15	1.00	(0.99,1.00)	0.582

TAB. 3 CYTOKINES INVOLVED IN IRI/AKI GROUPS P<0.01

IRI+AKI+	**IRI+AKI-**	**IRI-AKI+**	**IRI-AKI-**
G-SCF	IP-10	TNFa	IP-10
IL1RA	HSP90a	IL - 10	Eotaxin
IL-6	___	___	sCD26

6 Discussion:

Ischemic reperfusion injury is an inevitable process which starts with cross clamp at donor side and continues after liver reperfusion. All data until now suggests that the main role in this process is hypoxic cellular stress and subsequent inflammation-mediated injury. After reperfusion, IRI doesn't go away with time, but can increase in severity and could cause primary non function, delayed graft function or graft lost. The immune system plays a central role in this damage. The effect of IRI is not only localized to the liver, it's also generalized involving other organs. One of the principle mediators of IRI are cytokines and chemokines. They've been produced in the liver during the cold ischemia and during reperfusion were released into the blood circulation. From the evolutionary perspective the role of cytokines is to prepare the human body to survive trauma, shock or severe inflammation such as sepsis. In organ transplant their role is controversial and mostly negative. This can be explained with the fact that on the evolutional scale, transplantation has been done for less than a hundred years and so the human body doesn't know how to accommodate this new challenge. It is done with a "cytokine storm" which is always counterproductive and harmful. This cytokine

“storm” is responsible for all general effects of IRI. For instance, post reperfusion syndrome has been widely seen after liver reperfusion. In our case series we found it in almost all cases with IRI+AKI+ patients, despite the good anesthesia pre work up. The patients from that group received 2 or more vaso active pressors in order to keep BP in standard parameters. This led to subsequent kidney, lung and heart injury. IRI is also tightly related with the quality of the liver. Fatty livers are prone to ischemic injury. In our case series half of the patient in the IRI+ group had macrosteatosis greater than 20%. All hepatocytes with macrosteatotic inclusions will not survive the organ reperfusion and will be lost. Hepatic steatosis along with fibrosis are the two most important predictable liver quality factors. In our series, livers with macro steatosis greater than 20% were associated with severe ischemic reperfusion injury. The second factor along with steatosis involved in IRI was the cold ischemia. This finding is just common sense as in all transplantable organs and tissue, the more time a liver spends in ice, the more hepatocytes will be necrotic. The liver resilience to cold ischemia is tightly connected with the quality of the liver. Livers from young and lean donors without steatosis or fibrosis can stay in ice for 10/12 hours without any major post-reperfusion repercussions. In terms of kidney injury we found out that two cytokines TNFa and IL-10 are much more elevated in IRI+AKI+ group.

In this study we tried to individuate which cyto or chemokine is mainly involved in ischemic reperfusion injury. Circulating cytokines and chemokines are differentially expressed in IRI+ and IRI– patients before and after OLT. We used unsupervised hierarchical clustering to first identify patterns before and after transplant of 40 cytokines, chemokines, and growth factors in the systemic blood (Tab. 2). We found

a pattern of cyto/chemokines presented in all patients with IRI. G-SCF, IL1RA, IL-6 and HSP90a have been statistically elevated in IRI+ group (Tab. 3). In patients without IRI we found that several cyto/chemokines have been elevated – Eotaxin and sCD26. These findings suggest that those cyto/chemokines may have some protective characteristics. Interestingly, IP-10 was found to be elevated in both IRI positive and negative group. This confirms the fact that some cytokines can play on both sides of the ischemic reperfusion process as catalytic or inhibitor. The relationship between these cytokines and IRI is represented on fig. 1.

FIG. 1 BOX PLOTS OF SEVERAL CYTO/CHEMOKINE LEVELS IN RELATION TO IRI

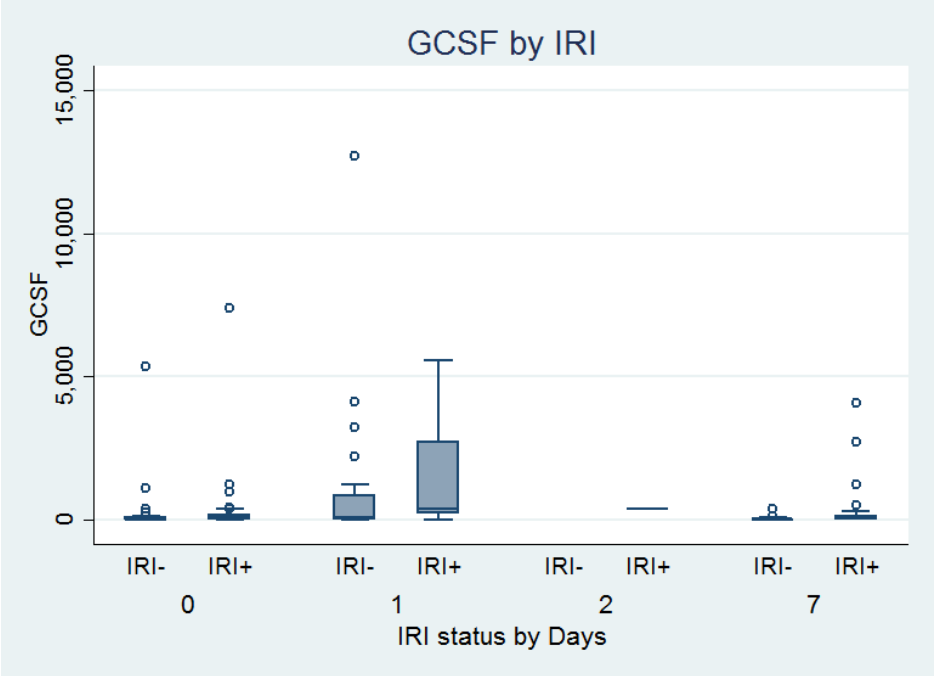


FIG. 2 BOX PLOTS OF SEVERAL CYTO/CHEMOKINE LEVELS IN RELATION TO IRI

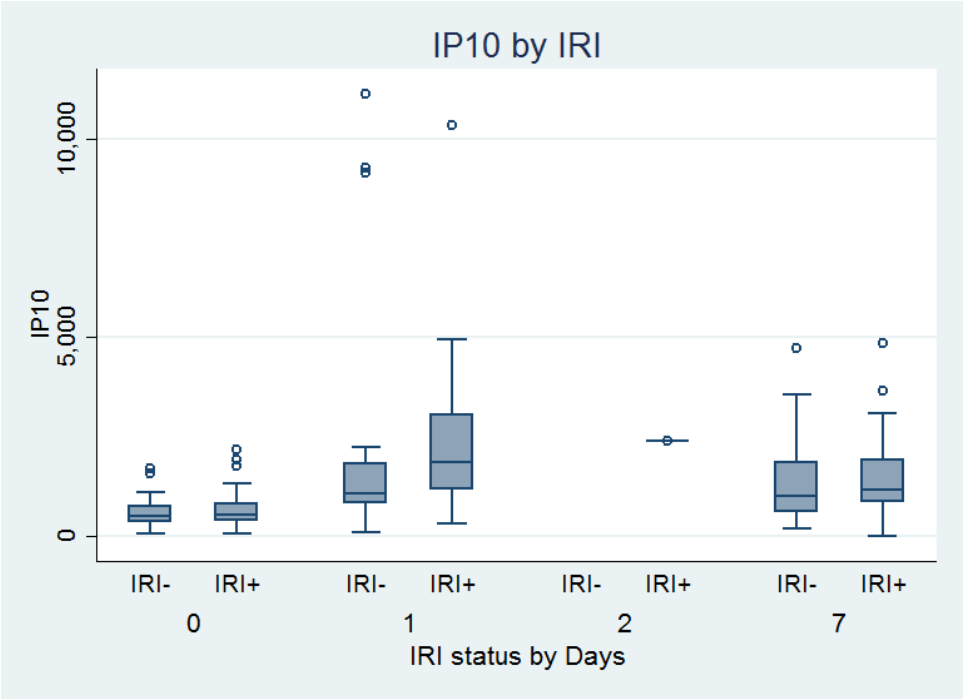


FIG. 3 BOX PLOTS OF SEVERAL CYTO/CHEMOKINE LEVELS IN RELATION TO IRI

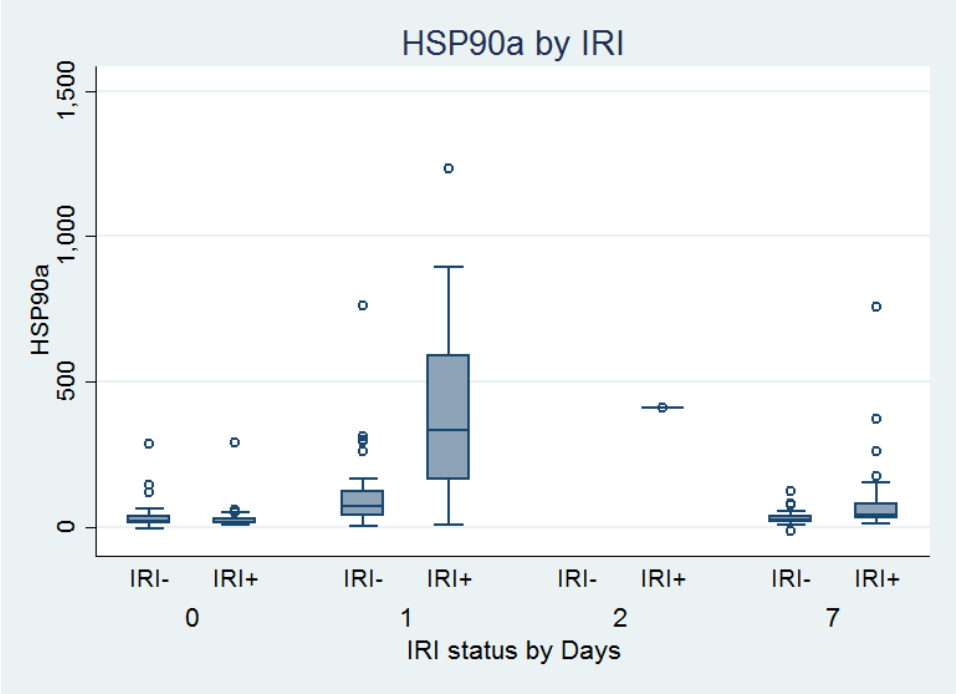
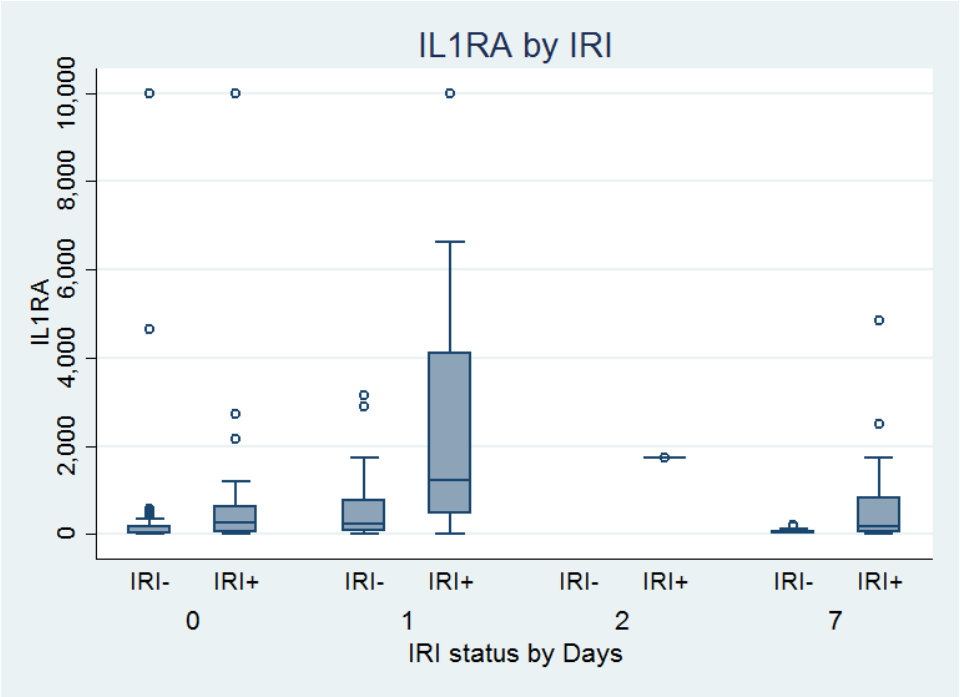


FIG. 4 BOX PLOTS OF SEVERAL CYTO/CHEMOKINE LEVELS IN RELATION TO IRI



Severe IRI occurs at several key time points during the process of OLT, starting with oxidative stress in the organ donor, continuing during cold storage and transport of the organ, and again when the organ is warmed and reperfused with blood from the recipient. These events all contribute to the overall degree of IRI and provide a plethora of signals to and from both the donor and the recipient's immune systems. This ultimately results in immune cell recruitment and activation to the site of injury. Therefore, defining the qualitative and quantitative immune events of IRI in human OLT is critical to the development of targeted therapies as well as improvement of overall OLT outcome. Here, we examined the evolution of the immune response in OLT recipients in the context of IRI, with the overall goal of determining cytokine

and chemokine profiles that could potentially be used to assess the immune status of the OLT recipient and risk of IRI. IRI is also accompanied by distinct innate and adaptive immune cytokine signatures before and after transplant. The liver is a prominent cytokine target due to its key role in the acute phase response. our study shows that IRI+ patients have a distinct cytokine signature compared with IRI– patients already at the time of transplant surgery, suggesting a specific immune phenotype that might predispose an OLT recipient to IRI. This signature comprises elevated levels of TNF-α, IL-5, IL-13, IL-2, CXCL8, IL-7, IL-1Ra, and EGF.

At the end, the final goal would be to identify potential pathways for therapeutic intervention in order to prevent or minimize the development of ischemic reperfusion injury and thereby improve post-transplant graft outcomes.

Chapter IV

Future perspective

Since the first liver transplant done by Starzl in 1967, a lot has changed. Yet the major hurdle remains ischemic reperfusion injury and all the consequences that it brings. In the last ten years different teams all over the world have been trying to mitigate IRI with different methods. Still there is no unique solution to this complex issue, although some study groups have come close to it. Here I'll try to synthetize what was done until now as treatment options in IRI.

1 Pharmacological treatment options used to reduce ischemia reperfusion injury

Hepatic IRI is the main cause of morbidity and mortality in liver surgery and transplantation (1, 2). Thus preventing it remains the main goal of every transplant center

Alfa tocopherol

Alfa tocopherol is an effective inhibitor of the free radical chain reaction of lipid peroxidation, and also it acts as inhibitor of protein kinase C (3). In different animal studies it was shown to protect the liver against lipid peroxidation and enhancing endogenous antioxidant activity also reducing the level of mitochondrial damage associated with oxidative stress. In addiction Alpha-tocopherol has also shown beneficial effects in cold IRI (4).

Ascorbic acid

Ascorbic acid is an important antioxidant with strong reducing and ROS scavenging properties (5). Studies in rats showed that low doses of ascorbic acid (100 mg/kg) have protective effects in hepatic function while high doses (1000 mg/kg) have been reported to have a prooxidant effect and aggravated the injury likely induced by increased reduction of ferric iron to the ferrous form (6).

Alpha lipoic acid (ALA)

Alpha lipoic acid (ALA) several studies have found that ALA acts on oxidative stress by scavenging free radicals eliminating the radical effects of ROS (7). Studies on the rats have shown that pretreatment by ALA improved tolerance to ischemia. Study performed on twenty-four patients subjected to preconditioning with ALA intravenously 15 min before inflow occlusion of the liver has shown that serum levels of aspartate transferase and alanine transferase were reduced (8).

Melatonin

Melatonin is a hormone produced by the pineal gland that helps regulate circadian rhythms and it has been shown to exhibit antioxidant activity. In humans, the endogenous level of melatonin follows a circadian rhythm with an increase in secretion soon after the onset of darkness and a peak between 2 and 4 am. Multiple studies have been done on rodents and humans in settings of different organ transplant – lungs, kidneys, bone marrow, liver, pancreas etc. It is one of the most prominent single agents in treatment of liver IRI. Melatonin as a pretreatment in donor surgery induces a decrease of TNF-α and intercellular adhesion molecule 1 (ICAM1) levels with a significant attenuation of hepatic leukocyte infiltration,

vacuolization, and cell death. In liver transplant patients it was associated with lower post LT transaminase and less ICU stay. Of note, the dose used in organ transplantation is between 1000- and 3000-fold difference compared with melatonin dose for sleep or jet lag. In conclusion, the usage of melatonin in liver transplantation may be a useful therapeutic tool in the complex treatment of patients with end stage liver failure, although more human data needed.

Desferoxamine

Desferoxamine is an iron chelator used in thalassemia and it can promote lipid peroxidation and attenuates hepatic microvascular injury (9) Experimental studies during canine liver transplantation using desferoxamine pretreatment, have shown beneficial effects in warm and cold hepatic ischemia (10).

Trimetazidine (TMZ)

Trimetazidine (TMZ) is a drug used in ischemic heart disease thanks to its cytoprotective properties. As shown in several studies, the mechanism of TMZ interacts with mitochondria, chelation of metals, energy metabolism, oxidative stress and microcirculation (11). Precisely it has been shown in an experimental model of partial hepatectomy under hepatic blood inflow occlusion that TMZ reduced liver injury, improved liver regeneration and survival rate (12).

N-Acetylcysteine (NAC)

N-Acetylcysteine (NAC) is glutathione precursors and it is currently the drug of choice in the treatment of fulminant liver failure due to paracetamol overdose. Clinical trials with NAC in patients undergoing liver transplantation found that it was associated with less hepatocellular injury, better liver function and lower incidence of primary graft dysfunction (13). In the pediatric liver transplant population, the usage of NAC was associated with lower ALT levels
And lower postoperative in-hospital stay, while rejection was less severe (14).
Multiple drugs and molecules have been used in animal clinical studies. Drugs such as – allopurinol, erythropoietin, bucillamine, idebenone etc all showed some positive effect on IRI. The common thing between all of them was that they provide only partial protection from IRI and have never been tested in human clinical trials.

2 Surgical preconditioning

In 1986, ischemic preconditioning (IP) was first reported by Murry et al in a canine model. The study was done by pre-exposing the heart to a brief period of ischemia and then reperfusion before the actual period of ischemia. The effect was evaluated and confirmed in different organs such as kidney, liver, muscle flaps and lungs.

Ischemic preconditioning of the liver involves a brief period of portal triad clamping usually between 5–15 minutes followed by a brief period of reperfusion (10–20 min) before a prolonged period of ischemia. The exact mode of action of IP in the prevention of post-operative hepatic complication has not yet been fully

understood. Studies and observation in animal models showed that IP lowers the levels of mitochondrial ROS and thus reduces the oxidative stress- mediated damage in liver IR injury and improves hepatic microcirculation (15). The protective effect of IP in liver transplants against hepatic IRI induced damage has also been attributed to its conservative action on hepatic ATP, tolerance to mitochondrial permeability transition and preservation of ATP synthase activity and thus tolerating the IRI- induced lowering of ATP. Earlier study on IP has reported anti-inflammatory action in liver transplants by inducing the expression level of adiponectin which inhibit NFκB inflammatory cascade. In addition, a suppressive effect on TNF–α expression and serum levels of interleukin-6 have been reported for the positive effects in liver graft survival (16). In mid-2000, Koneru at al published the first IP experience in human liver transplantation. After this study other centers began their experience with IP on the donor side. It was found that the post-transplant transaminase release (AST and ALT) was significantly reduced, especially in marginal grafts treated by IP, and the severity of histological IRI was improved. However, no difference was observed in functional recovery or incidence of delayed graft function, although a trend towards better graft and patient survival was observed in a short-term follow-up of 6 months (17). Finally, the latest meta-analysis in usage of IP in liver transplantation detected no improvement in post-transplant transaminase release, no differences in PNF, mortality, or retransplantation. Authors concluded that there is no evidence to support or to refute the use of IP during liver procurement (18).

3 Hypothermic machine perfusion - HMP

HMP was proposed by Belzer in the early 1960s and first performed by the group of Guarrera et al. It preserves the organ by providing a constant perfusion through the organ's blood vessels with a machine perfusion solution (MPS), which is usually perfused with modified UW or HTK solution. In the kidney transplant field HMP became a standard for organ preservation (LifePort), since its superior in terms of organ preservation compared to static cold preservation. In liver transplantation, the use of HMP is still debatable. The function of HMP is simply hypothermia causing decreased cellular metabolism rates due to slowing down of enzymatic processes of multiple proteins in the cold. However, cold storage of organs without active supply of oxygen and nutrients is limited to the energetic reserve of liver grafts. These are depleted between 12 and 24h of storage as anaerobic glycolysis is the main metabolic pathway. This leads slowly to intracellular acidosis and nucleotide depletion. The time an organ can sustain these conditions therefore depends on cooling to reduce metabolic activity and oxygen requirements as well as the use of fluids designed to preserve the intracellular homeostasis in the absence of proper Na+/K+ pump function. The oxygenation of cells through HMP leads to a very limited ROS release. At the same time, forward metabolism of accumulated succinate leads to ATP resynthesis. The phenomenon of shutting down most fueling processes and supporting mitochondrial is probably related to a common ancestral process in animals, humans, and plants, enabling cells to survive in winter time by hibernation or winter rest. Reperfusion of ischemic livers treated by cold oxygenated perfusion, triggers significantly less oxidative damage in mitochondria with subsequent less downstream inflammation (19). Importantly,

mitochondrial switch from ischemic to fully ATP loaded status needs 1–2 h of cold oxygenated perfusion, which can be performed after cold storage in transplant centers. The disadvantage of this approach is the current lack of methods in testing the energetic status in perfused livers. It is also unclear how long cold oxygenated perfusion could be safely maintained. Another hurdle is the lack of parameters to assess the liver function (as lactate or bile production).

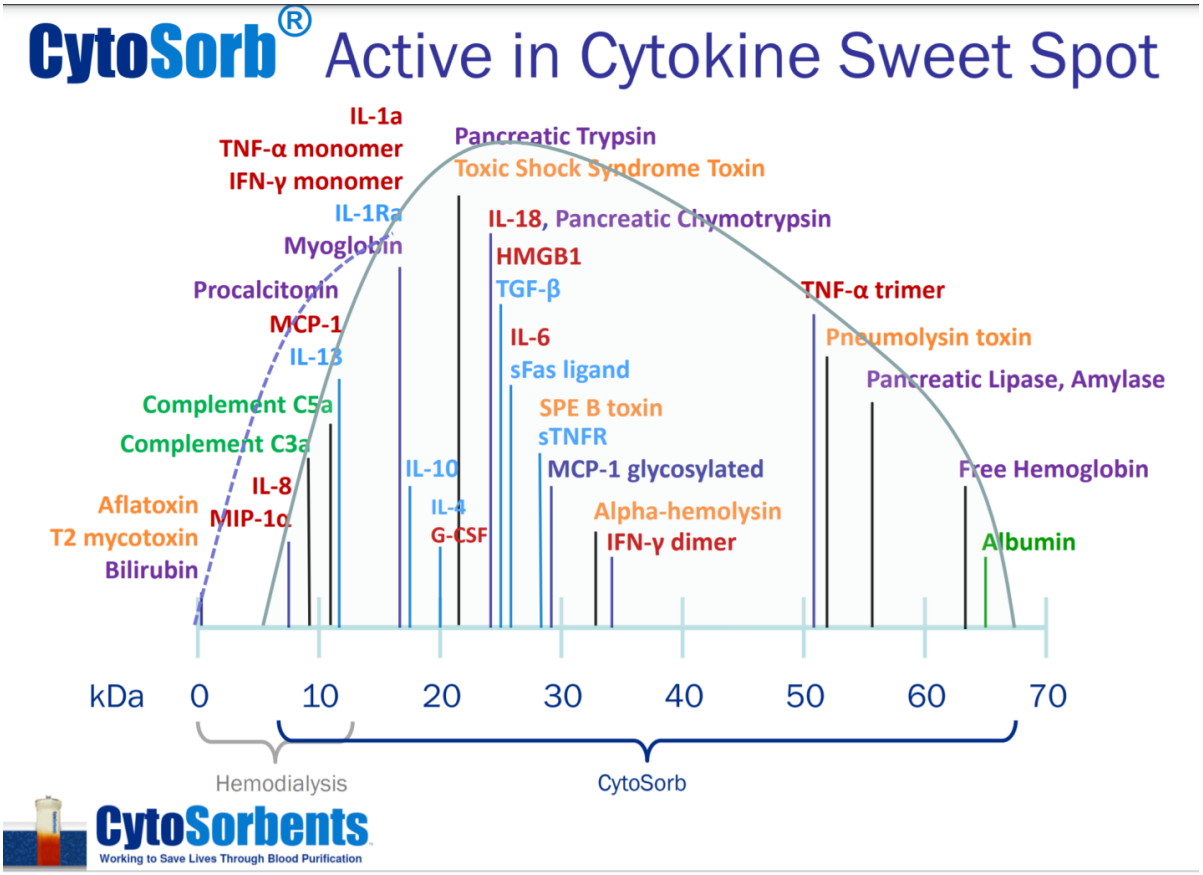
In 2009 Guarrera at al. performed the first prospective liver HMP study in humans providing safe and reliable preservation of donor livers. Since then multiple centers worldwide adopted this technique. Results of these clinical trials shows that HMP significantly reduces molecular markers of I/R injury including expression of proinflammatory cytokines, adhesion molecules, and migration of leukocytes (20). The Zurich group has been applying oxygenated hypothermic machine perfusion at the end of cold storage (called HOPE study) to recondition liver grafts after DCD donation. Reduction of hepatocyte necrosis and transaminase release, recovery of ATP content and increased bile production in all livers treated with HMP was observed.

However, HMP does not seem to couple better preservation with gain of function, despite the re-balanced energetic status. HMP applicability in liver transplantation remains unclear and large-size randomized clinical trials are required to further elucidate the potential of this new preservation technique.

4 Cytosorb as a treatment option in liver transplant ischemic reperfusion injury.

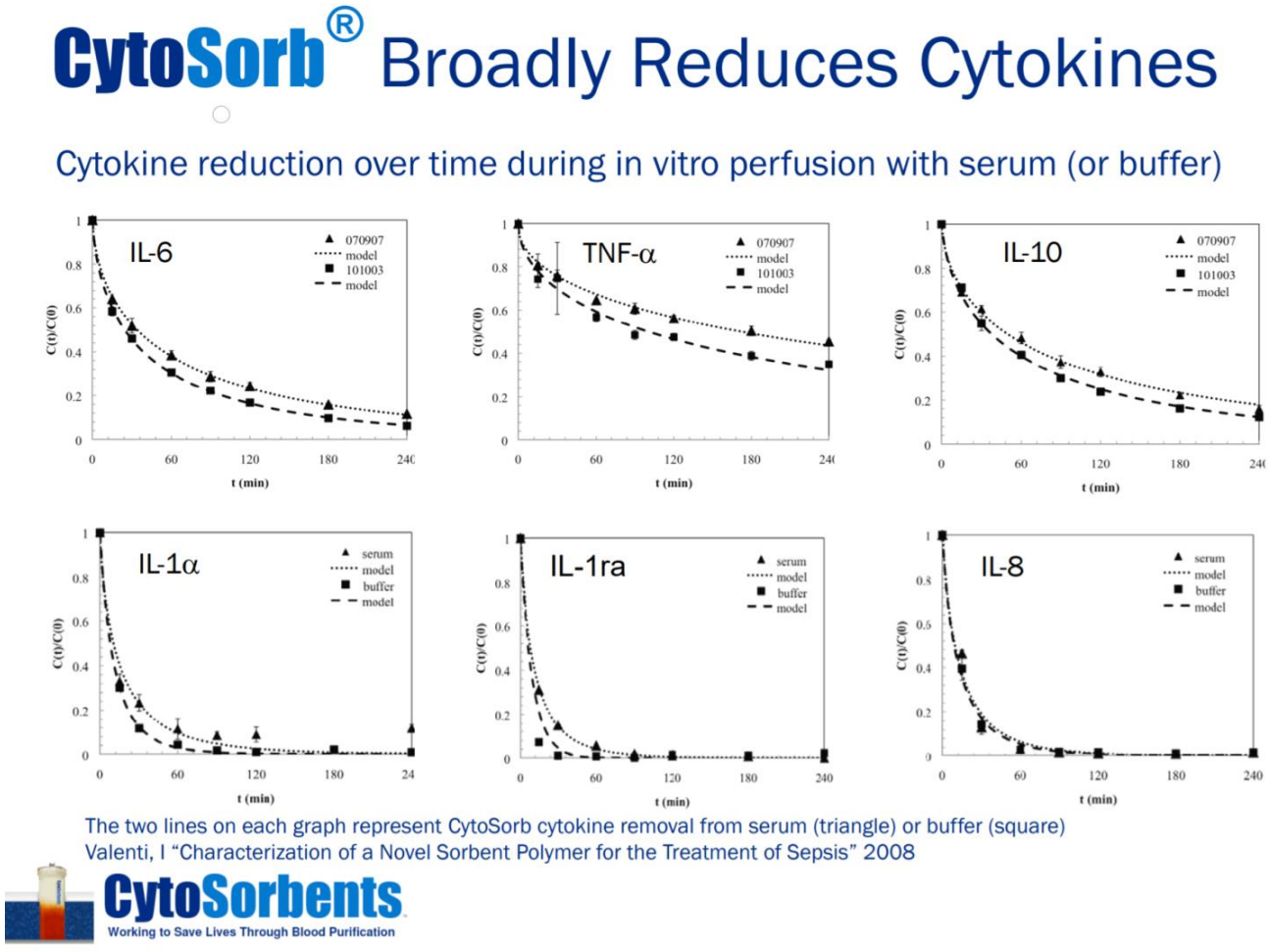
The CytoSorb therapy is based on extracorporeal blood purification that effectively reduces excessive levels of inflammatory mediators. It is able to remove molecules in the 5–60 kDa range (fig 1) which comprises the majority of inflammatory mediators and some endogenous molecules (myoglobin, free hemoglobin, bilirubin, and bile acids), and some exogenous molecules (toxin, metals, and drugs) also fall within this size spectrum. In doing so, the goal is to reduce the overshooting systemic inflammatory response while the physiological immune response is maintained.

FIG. 1 THE ABSORPTION RANGE OF CYTOSORB



This absorber is composed of biocompatible porous polymer beads made of polystyrene-divinylbenzene, that act like tiny sponges to remove substances from whole blood based on pore capture and surface adsorption. The Cytosorb modulates the immune response throughout - effective reduction of excessive cytokine levels (fig. 2), decreased de novo synthesis of inflammatory mediators, controlled attenuation of the overshooting immune response and re-targeting of the cellular immune defense to the focus of infection. It is the standard of care in septic shock, cardiac surgery (used with ECMO), combined in renal replacement therapy as well as alone. Recently it's been FDA approved in treatment of Covid-19.

FIG. 2 REDUCTION OF THE LEVEL OF DIFFERENT CYTOKINES OVER TIME DURING CYTOSORB THERAPY



Limited data has been published on Cytosorb usage in liver failure and liver transplantation. In settings of acute liver failure Cytosorb showed notable success (21 - 23). In 2020 Tomescu et al. (24) published the first prospective case series of patients with acute liver failure (ALF) treated with Cytosorb. The aim of this study was to assess the clinical effects of a hemoadsorption column on biochemical parameters in patients with ALF. They observed an improvement in liver function tests and a decrease in C-reactive protein, as well as reduced ICU and hospital stay. So far Cytosorb is the only treatment available on the market for physical removal of cyto and chemokines.

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CHAPTER V

Conclusions

Performing organ transplantation inevitably leads to ischemic reperfusion injury, which is a potential threat to the success of the procedure. Different approaches have been developed to reduce the complex pathogenesis of IRI. So far there are three distinguished approaches. The first one is preconditioning which can be done surgically or pharmacologically. As described above this has been done in different settings (in lab animals as well as clinical studies on humans). The main issue remains the inconsistency of the results and the fact that IRI occurs independently of the preconditioned treatment. Another weak point in pharmacological preconditioning is the fact that it cannot be used in DCD settings (since it is forbidden to administer any drug except heparin before withdrawing life support). Also, it is still unclear how different substances can interact with the recipient's immune system.

The second way to reduce and potentially prevent ischemic reperfusion injury is the usage of perfusion systems. There are currently two types of perfusion systems – cold and normo perfusion. Mechanical perfusion systems not only represent a valid alternative to the standard static cold storage, but also allow evaluation of graft viability before transplantation and might recover the injury induced by ischemia. Furthermore, as described from Zhao et al., NMP can be used for so called *ischemia-free liver procurement*. Zhao performed for the first time the transaction of a liver between donor and recipient without interrupting the blood flow to the

organ. Thus, there was no ischemic reperfusion injury. This technique can be used for all marginal DBD livers where severe IRI is expected. This is a newly re-born area of interest, which needs further exploration before drawing ultimate conclusions. Currently available data seems to point towards a more efficient preservation of grafts with a normo perfusion system. Furthermore, it offers the unique opportunity to treat the organ with novel therapies, converting high-risk suboptimal grafts into transplantable livers. If reconditioning of risky grafts would successfully translate into clinical practice, it will certainly represent a major progress in transplantation. This offers a valuable tool to help rebalance the impaired equilibrium between donor offer and transplant demand.

A third way to cope with IRI is Cytosorb usage. This treatment is reserved for those patients with IRI already established. Since this device functions as an absorber, it can remove from the blood stream almost all cyto and chemokines produced during IRI after liver transplant. As we have come to understand, the majority of these vaso active substances are produced during the first 48/72 h and the usual treatment with Cytosorb shouldn't exceed three days. As it is now the major hurdle in the utilization of Cytosorb in liver transplant is the lack of data. Although Cytosorb is a well-established treatment for patients in septic shock it is still unknown the benefit of such treatment in liver transplantation.

In conclusion, multifactorial and pleiotropic approaches have been advocated for simultaneous action on several IRI pathologies. Taking in consideration all available methods to minimize ischemic reperfusion injury after liver transplant, the one that stands out is the normo perfusion system. As part of the OrganOx study we showed

that this method is safe, can diminish IRI, but most importantly can be used to assess organ viability before the actual transplant. Thus, minimizing the chance of a primary non functioning graft.