CATÓLICA
FACULTY OF BIOTECHNOLOGY
PORTO

Improving Organ Quality through Perfusion During Transplant Surgery

By
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Improving Organ Quality through Perfusion During Transplant Surgery

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science Degree in Biomedical Engineering

By
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Este trabalho é dedicado à minha família, em especial aos meus pais e ao Pedro – por todo o apoio, carinho e amor incondicional.

"Science and everyday life cannot and should not be separated."

- Rosalind Franklin

"It had long since come to my attention that people of accomplishment rarely sat back and let things happen to them. They went out and happened to things."

- Leonardo da Vinci

RESUMO

Atualmente, a área da transplantação de órgãos enfrenta uma crítica escassez de órgãos viáveis para transplante, o que afeta significativamente o prognóstico dos pacientes em todo o mundo. Como tal, é necessário aumentar o conjunto de potenciais dadores, o que pode ser feito através da utilização de dadores de critérios expandidos (*Expanded Criteria Donors* - ECDs), o que permite aumentar o grupo de potenciais dadores e diminuir os tempos de espera por transplantes. Estes dadores são geralmente pessoas com mais de 60 anos ou 50 anos com pelo menos dois fatores de risco. Contudo, o uso de órgãos de ECDs pode aumentar a possibilidade do risco de síndrome de reperfusão (IRI), devido às características particulares destes órgãos, tornando-os mais suscetíveis a certos danos e menos resistentes ao IRI, sendo esta a problemática mais comum associada ao transplante de fígado.

Esta tese aborda destaca novas abordagens para a preservação de órgãos, com o intuito de melhorar a eficácia dos transplantes quando se recorre a ECDs. Assim, é feita uma avaliação abrangente das soluções de preservação existentes, bem como dos métodos de preservação atuais, tais como *Static Cold Storage* (SCS), *Normothermic Machine Perfusion* (NMP) e *Subnormothermic Machine Perfusion* (SNMP), com particular ênfase para *Hypothermic Oxygenated Machine Perfusion* (HOPE). Neste estudo, o HOPE aplicado como o melhor método de preservação de órgãos devido às suas vantagens únicas, tais como melhorar a função mitocondrial e, portanto, reduzir a vulnerabilidade do fígado ao IRI. Assim, o objetivo desta tese é avaliar a eficácia do HOPE no contexto de cirurgias pós-transplante, através da análise quantitativa do Mononucleotídeo de Flavina (FMN) como um biomarcador preditivo da viabilidade hepática. Foram desenvolvidos meticulosamente protocolos, sendo estes implementados dentro do ambiente hospitalar para garantir que o estudo seguisse os parâmetros pré-determinados. Foram recolhidas informações sobre os dadores e recetores, sendo estas incorporadas num conjunto de dados abrangente relativa a seis transplantes de fígado. Em última análise, foi estabelecida uma correlação entre os dados do órgão do dador, os resultados do recetor, mais especificamente a sua análise clínica, e os níveis de FMN.

Os resultados indicam que a análise do FMN tem potencial de ser um indicador substancial da viabilidade do órgão, fornecendo à equipe de transplante uma abordagem quantitativa adicional que pode auxiliar na tomada de decisão. Esta tese enfatiza a importância crítica para os hospitais implementem análise de FMN como um marcador de viabilidade de órgãos em tempo real, melhorando as taxas de sucesso do transplante e proporcionando uma vantagem competitiva na otimização do uso de ECDs.

Palavras-chave: Transplante hepático, Dadores de critérios expandidos, *Hypothermic Oxygenated Machine Perfusion*, Avaliação de viabilidade.

ABSTRACT

Presently, the field of organ transplantation is confronted with a critical scarcity of viable organs, which has a deep impact on the prognosis of patients across the globe. Through the implementation of the Expanded Criteria Donors (ECDs), where organs from donors older than 60 years or older than 50 years with at least two associated risk factors are used for transplantation, it is possible to increase the pool of donors with the additional incentive of earlier transplantation. Nevertheless, the risk of ischemia-reperfusion injury (IRI) may also be increased by the use of ECDs due to the suboptimal status of these organs, leaving them more susceptible to damage and less resilient to the stress of the reperfusion process, which is a major concern in liver transplantation.

Consequently, the present thesis overviews novel approaches for organ preservation in order to improve transplants efficacy when using ECDs. A comprehensive evaluation of the current perfusion solutions and organ preservation methods are presented, such as Static Cold Storage (SCS), Normothermic Machine Perfusion (NMP) and Subnormothermic Machine Perfusion (SNMP), with particular emphasis to Hypothermic Oxygenated Machine Perfusion (HOPE). HOPE was applied in this study as organ preservation method due to its unique advantages, such as enhancing mitochondrial function and therefore reducing the liver's vulnerability to ischemic injury. Thus, the aim of this thesis is to evaluate the efficacy of HOPE in the context of post-transplant surgeries, assessed through the quantitative analysis of Flavin Mononucleotide (FMN) as a prospective biomarker of liver viability. Protocols were meticulously developed and implemented within the hospital setting to ensure that the study followed pre-determined parameters. Donor and recipient information were incorporated into a comprehensive dataset derived from the analysis of six liver transplants. Ultimately, a correlation was established between donor organ data, recipient outcomes, more specifically their clinical analysis, and FMN levels.

The findings suggest that FMN analysis has the potential to be a substantial indicator of organ viability, providing the transplant team an additional quantitative approach that can assist in their decision-making. This thesis emphasizes the critical importance for hospitals to implement FMN analysis as a real-time organ viability evaluator, improving transplant success rates and providing a competitive advantage in optimizing the use of ECDs.

Keywords: Liver transplantation, Expanded Criteria Donors, Hypothermic Oxygenated Machine Perfusion, Viability Assessment.

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SYMBOLS AND ABBREVIATIONS

ALT – Alanine Transaminase

AST – Aspartate Transaminase

ATP – Adenosine Triphosphate

BMI – Body Mass Index

CHUdSA – Centro Hospitalar Universitário de Santo António

DAMPs – Danger Associated Molecular Patterns

DCD – Donation After Circulatory Death

DNF – Delayed Graft Function

ECDs – Expanded Criteria Donors

ESB-UCP – Escola Superior de Biotecnologia da Universidade Católica Portuguesa

FMN – Flavin Mononucleotide

HOPE – Hypothermic Oxygenated Machine Perfusion

HTK – Histidine-Tryptophan-Ketoglutarate

ICU – Intensive Care Unit

IRI – Ischemia-Reperfusion-Injury

NADH – Nicotinamide Adenine Dinucleotide (Hydrogen)

NMP – Normothermic Machine Perfusion

OT – Organ Transplantation

OR – Operating Room

PNF – Primary Nonfunction

ROS – Reactive Oxygen Species

SCS – Static Cold Storage

SNMP – Subnormothermic Machine Perfusion

UW – University of Winsconsin

1 STATE OF THE ART

1.1 CURRENT STATUS IN ORGAN TRANSPLANTATION

Presently, organ transplantation (OT) stands as the paramount therapeutic option for patients with end-stage organ failure (Blondeel et al., 2023). Since it offers improved outcomes in terms of patient mortality and morbidity, this sort of treatment might be considered one of the most notable innovations in modern medicine (Bezinover & Saner, 2019). Over the years, there have been several breakthroughs in medicine that positively impacted the field of OT. Developments on immunosuppressive medicine, surgery, organ preservation and post-transplantation care, allowed to significantly improve OT outcomes (Jing et al., 2018). However, it still faces many challenges, being the organ shortage crisis the most prominent (Bezinover & Saner, 2019).

1.1.1. Organ Shortage Crisis

Even though medical progress has been unprecedented in this era, the organ scarcity crisis represents one of the most pressing challenges in modern medicine (Abouna, 2008).

Annually, numerous individuals await life-saving transplantations, anticipating a renewed opportunity for survival. However, the escalating demand for organs in comparison to those that are available for transplantation continues to rise (Blondeel et al., 2023).

In Portugal, the kidney ranks as the organ with the highest transplant rate, and the liver as the second one (Luz, 2023b). In 2022, there were 49 kidney transplants per million population (pmp), marking an increase from 42.1 pmp in the previous year (Luz, 2023b). Liver transplants followed with 20 pmp, making them the second most common procedure in 2022. Other transplants in the same year included lungs at 3.9 pmp, hearts at 3.7 pmp, and pancreases at 2.5 pmp (Luz, 2023b).

As of the latest data, 2,546 patients are on the waiting list for a kidney transplant in Portugal, followed by 258 for the liver (Luz, 2023a). There are 121 patients awaiting a lung transplant, 98 for the heart, and 62 for the pancreas (Luz, 2023a).

According to the *Instituto Português do Sangue e da Transplantação*, there was a significant rise in organ collections between 2020 and 2022. In 2020, 750 organs were collected, 872 in 2021, and 894 in 2022 (*IPST, IP - Dados Estatísticos - Transplantação*, 2023). However, when examining the organs that were actually transplanted, only 774 of the 894 were utilized (*IPST, IP - Dados Estatísticos - Transplantação*, 2023). This represents an 87% utilization rate, with the remaining 13% being discarded.

Considering these statistics, it is imperative to investigate for novel strategies and solutions aimed at mitigating the disparity between the quantity of organs available for transplantation and those effectively transplanted (Abouna, 2008). A promising approach has been the expansion of the donor pool with the use of Expanded Criteria Donors (ECD). Additionally, recent developments in organ preservation involve the use of machine perfusion, sustaining organ viability for extended periods of time and increasing the feasibility of organ transportation over longer distances (Blondeel et al., 2023; Del Prete et al., 2022; Jing et al., 2018). By combining our efforts with the advancements in the biomedical engineering field, we can help reduce organ scarcity and provide patients with improved health care and longevity.

1.2 EXPANDED CRITERIA DONORS (ECDS)

It has become evident that innovative solutions are needed to close the gap between supply and demand in the organ scarcity dilemma. Among all the approaches that have been proposed, using Expanded Criteria Donors (ECDs) to increase the donor pool offers a feasible solution to address the deficit (Blondeel et al., 2023). Through this strategy, organs that were previously untapped gain the capacity for exploration, changing the paradigm of organ donation and transplantation.

ECDs include donors who do not meet the standard criteria established to ascertain optimal donor quality due to a variety of physiological, medical, or donation-related factors (Blondeel et al., 2023). The use of this type of criteria expands the pool of potential donors, but also

highlights the need to measure the advantages of using a wider range of organs against the risks and difficulties associated with ECDs.

The criteria for categorizing ECDs in organ transplantation vary across different hospitals, reflecting the complex nature of such evaluations. However, the recognition of the need for a wider consensus has given rise to initiatives such as the consensus meeting held in Paris in March 2007 (Durand et al., 2008). The fact that a wide range of specialists from Asia, the United States and Europe convened to discuss the concept of ECDs made this gathering noteworthy (Durand et al., 2008). It is also important to highlight that organizations such as Eurotransplant provide recommendations that act as a reference framework to help standardize these requirements (Widmer et al., 2022).

The use of ECDs combined with dynamic organ preservation techniques offer an extra opportunity to safely increase the pool of transplanted organs, with the possibility to assess their viability prior to implantation (Widmer et al., 2022). Despite the advancements in dynamic organ preservation techniques and the use of ECDs contributing significantly to the expansion of the donor pool, there remains an essential reliance on the utilization of marginal livers, underscoring the need to balance innovative practices with the practicalities of organ availability and suitability. Nevertheless, the utilization of "marginal" livers is possible and highly variable between hospitals, being influenced by risk strategies, donation rates, and surgical expertise (Karangwa et al., 2020). Thus, the ECDs that require particular attention in order to avoid negligent use of transplanted livers, include:

Steatosis

Steatosis, commonly known as fatty liver disease, is a medical condition characterized by the accumulation of excess fat in liver cells (*Steatosis: Definition, Symptoms, Treatment, and More*, 2022). Steatosis can be divided into three categories: severe (>60%), moderate (30%-60%) and mild/minor (30%) (Widmer et al., 2022). Microvesicular steatosis is generally associated with a lower impact when it comes to ischemia-reperfusion injury, lower compromised function, and also a lower risk of early allograft dysfunction compared to macrovesicular steatosis. On the contrary, the use of moderate or severe appears to be more clinically significant due to its severity. There is a correlation between the use of livers with moderate steatosis and elevated incident of early allograft dysfunction, biliary complications, and reduced graft survival. On the other hand, livers with severe steatosis are associated with compromised function after the

operation, the requirement for renal replacement therapy, and also, lower rates of patient and graft survival (Patrono et al., 2023).

Overall, steatosis, particularly in marginal liver grafts, has been linked to an increased vulnerability to ischemia-reperfusion injury, premature functional failure and may have adverse effects on the regenerative capacity and functionality of the liver following the transplant procedure, as well as other severe complications due to its unfavourable metabolic state (Del Prete et al., 2022; Durand et al., 2008; Patrono et al., 2023).

Age

Elderly grafts often experience increased ischemia-reperfusion injury levels and a higher risk of complications following the liver transplant given their compromised ability to recuperate from metabolic insults (Widmer et al., 2022).

Age is a critical determinant due to its frequent association with decline in the resilience and regenerative capability of organs (Durand et al., 2008). Enhanced cellular and systemic indicators of deterioration and ageing in older organs may have an adverse effect on the survival and functionality of grafts following transplantation. In fact, most studies unequivocally prohibit the utilization of livers obtained from donors older than 60, 65, 70 or 80 years old (Widmer et al., 2022).

Liver Function

Liver function is assessed through blood tests that aim to identify and monitoring manifestations of liver injury. These tests can be performed to screen liver infections, including hepatitis. Overall, liver function tests measure the concentrations of specific proteins and enzymes in the blood, and abnormally high or low levels may indicate hepatic problems. Regarding these tests, measuring the levels of alanine and aspartate transaminases (ALT and AST) and total bilirubin are crucial (Lee et al., 2023; *Liver Function Tests - Mayo Clinic*, 2023). ALT is a liver-resident enzyme that facilitates the conversion of proteins to energy for liver cells. Elevated levels of ALT are secreted into circulation because of liver injury. The AST enzyme supports the digestion of amino acids, and like ALT, is typically found in small amounts in the blood. Elevated AST levels could potentially indicate liver injury, liver disease, or muscle damage (Lee et al., 2023; *Liver Function Tests - Mayo Clinic*, 2023). Bilirubin is a byproduct of the breakdown of red blood cells. Bilirubin is processed by the liver, and then excreted in bile. Elevated levels of bilirubin mean the bilirubin is not being processed and eliminated by the liver

which may indicate acute hepatic injury or pathology, and biliary duct obstruction (Lee et al., 2023; *Liver Function Tests - Mayo Clinic*, 2023).

Cold Ischemia Times

In the context of liver transplantation, cold ischemia time corresponds to the period during which a donor liver undergoes a cooling process subsequent to aortic clamping and initiation of *in situ* perfusion, prior to its rewarming process during implantation into the recipient. Prolonged cold ischemia constitutes a risk factor in the progression of delayed function and primary nonfunction of the allograft. While the precise mechanism remains uncertain, it is hypothesized that cold ischemia duration impacts graft function through its connection to ischemia-reperfusion injury (Busuttil et al., 2015; Durand et al., 2008; Widmer et al., 2022).

Donation After Circulatory Death

A promising method for increasing the organ supply is liver transplantation from non-beating heart donors, also known as Donation After Circulatory Death (DCD). In these cases, organs are extracted under controlled conditions following the certification of death, following an impasse period of two to five minutes (Durand et al., 2008; Widmer et al., 2022). This type of donors adds an additional level of complication because of the warm ischemia between circulatory arrest and organ preservation, which can potentially extend ischemic injury and influence the post-transplant outcomes of the patient. Additionally, it is critical to note that while their use is not yet possible in Portugal, this would be an enormous move forward for liver transplantation as it would substantially increase the pool of potential donors.

In relation to the subject of Expanded Criteria Donors, it is critical to emphasize the factors listed above and others, including body mass index (BMI), administration of vasopressors, and mechanical ventilation history (Durand et al., 2008; Widmer et al., 2022).

The implementation of ECDs has become a crucial approach in the field of organ transplantation in response to the increasing demand for donor organs. However, there are inherent difficulties associated with the use of ECDs, including the increased risk of transplant dysfunction and other possible complications. With the goal of minimizing these risks, optimizing the capabilities of ECDs, and enhancing transplant outcomes, it is imperative to implement the most efficient preservation techniques.

1.3 ISCHEMIA-REPERFUSION INJURY

Within the complex domain of organ transplantation, ischemia-reperfusion injury (IRI) is a pivotal occurrence that has a major impact on the results of transplant procedures. Ischemia-reperfusion injury (IRI) is a multifaceted and intricate occurrence that happens during liver transplantation, and it can be defined by a sequence of modifications at molecular, cellular and tissue levels. After a period of ischemia, in which the organ is deprived of oxygen and nutrients, IRI initiates when the blood supply of the organ is restored (Guibert et al., 2011a). Considering this, it is crucial to understand the mechanisms underlying IRI especially when addressing expanded criteria donors (ECDs), whose organs are more vulnerable to damage due to pre-existing conditions and prolonged ischemia durations.

Considering the definition above, IRI consists in a significant barrier, in particular on liver transplantation, which usually results in a variety of short-term and long-term complications that might have a significant impact on patient survival (Czigany et al., 2020). Intraoperative post-implantation hemodynamic instability (also known as post-reperfusion syndrome), delayed graft function (DGF) and primary nonfunction (PNF) are examples of complications that can be induced by IRI. Among these, PNF is considered the most severe, often culminating in patient mortality or a necessity of retransplantation (Czigany et al., 2020).

Mitochondrial dysfunction is the underlying cause of IRI as presented in Figure 1.

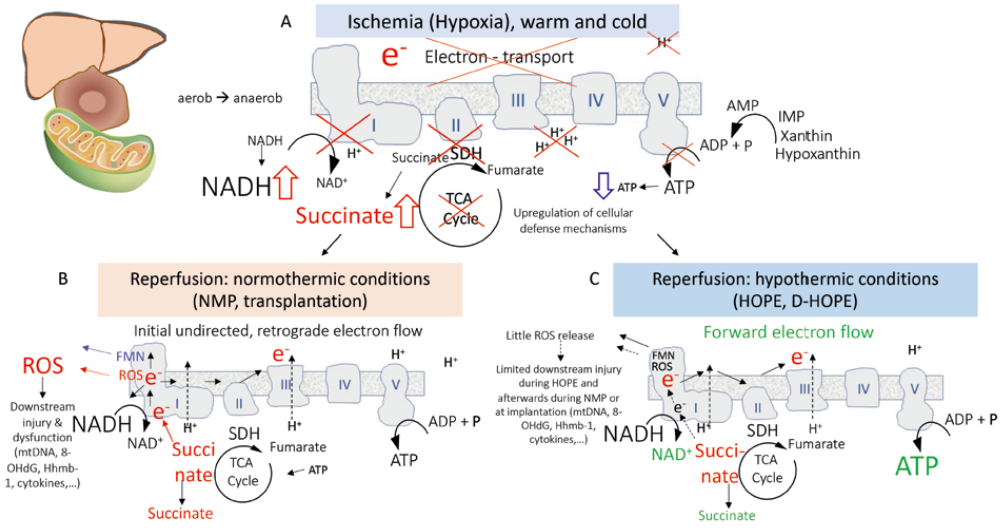

Figure 1 - Mechanisms of Ischemia-Reperfusion Injury (IRI) and protection through hypothermic oxygenated perfusion of liver grafts. Retrieved from (Del Prete et al., 2022).

In the process of producing energy, mitochondria, also known as the “powerhouse” of the cell, playing a significant role. This is mostly accomplished by oxidative phosphorylation, a process that results in the production of adenosine triphosphate (ATP), which is the primary energy currency of the cell (Blondeel et al., 2023). This process involves an electron transport chain positioned on the inner mitochondrial membrane, where electrons are transferred to oxygen via a succession of protein complexes, which results in the formation of a proton gradient crucial to produce ATP (Blondeel et al., 2023). This intricate procedure is significantly disrupted within the framework of IRI (Blondeel et al., 2023).

Due to the lack of oxygen, the electron transport chain is disrupted, which results in the accumulation of electron carriers such as NADH and succinate, which in turn obstructs the synthesis of ATP (Del Prete et al., 2022; Guibert et al., 2011a). In this way, cells are forced to engage in an anaerobic metabolism that is less effective, which ultimately results in the buildup of lactate and cellular acidosis. Deficiency in ATP has an impact on several cellular activities, including Na^+/K^+ ATPase, inducing cellular edema and electrolyte imbalance (Guibert et al., 2011a). These disturbances have the potential to induce cell death by apoptosis or necrosis. In addition to disrupting calcium homeostasis, the ischemic stage increases cellular damage and mitochondrial dysfunction (Del Prete et al., 2022; Guibert et al., 2011a).

A crucial component in this process is Flavin Mononucleotide (FMN), which functions as an intermediary electron acceptor within Complex I of the electron transport chain. During the ischemic phase, FMN has the potential to separate from Complex I, thereby affecting the effects of ischemia and intensifying the disrupting of electron flow (Blondeel et al., 2023).

After the ischemic phase, the reperfusion phase occurs, when oxygen supply is restored, and paradoxically causes more damage compared to the ischemic period (Blondeel et al., 2023). This process of reoxygenation causes a quick synthesis of Reactive Oxygen Species (ROS), where the dissociation of FMN has an essential role (Blondeel et al., 2023; Czigany et al., 2020; Patrono et al., 2023). Ultimately, this results in oxidative stress and activates the Mitochondrial Permeability Transition Pore (MPTP), which ultimately leads to cellular demise and release of pro-inflammatory molecules, such as Danger-Associated Molecular Patterns (DAMPs) (Del Prete et al., 2022; Schlegel et al., 2015). By activating hepatic immune cells that secrete cytokines, DAMPs induce a sterile inflammatory response that amplifies inflammation and attracts additional immune cells (Del Prete et al., 2022; Patrono et al., 2023; Schlegel et al., 2015).

When it comes to liver transplantation, the procedure of organ extraction, preservation, and implantation involves modifications that intensify IRI. The preservation of organs, which is commonly accomplished by cooling the organ in a specific solution, has an effect on metabolic pathways that contribute to the pathogenesis of IRI (Guibert et al., 2011a). It is important to note that the temperature during ischemia and reperfusion has a substantial impact on the level of mitochondrial damage. Specifically, normothermic reoxygenation causes more severe damage than hypothermic reoxygenation (Panconesi, Carvalho, et al., 2021).

1.4 ORGAN PRESERVATION TECHNIQUES

The preservation of donor organs is a fundamental component of organ transplantation, which has a significant influence on the success of the procedure, as well as on the patients' outcomes.

Traditionally, Static Cold Storage (SCS) has been the predominant technique utilized for organ preservation (Guibert et al., 2011a; Patrono et al., 2023). Dynamic preservation techniques have been created as a result of developments in the biomedical engineering field. These techniques include Hypothermic Oxygenated Machine Perfusion (HOPE), Normothermic Machine Perfusion (NMP) and Subnormothermic Machine Perfusion (SNMP). In fact, choosing the appropriate technique may increase the viability of organs, lessen the likelihood of post-transplant complications, and ultimately enhance patients' quality of life.

1.4.1. Traditional Organ Preservation Techniques

Static Cold Storage, also known as SCS, is an organ preservation technique that is predominantly used in the process of transplanting solid organs such as livers, kidneys, hearts, and lungs (Schlegel et al., 2015). Since it was first developed in the 1960s, SCS has evolved into the technology that is considered to be the standard for organ preservation (Jing et al., 2018).

Immediately after organ retrieval from the donor, the organ is cooled to a low temperature, which is normally anywhere between 0ºC and 4ºC, which reduces hepatic metabolism to

approximately 10% of its normal level (Blondeel et al., 2023; Schlegel et al., 2015). The main goal of SCS is to decrease the organ's metabolic activity, to minimize cellular damage, and also maintain its viability throughout the time period between organ retrieval and transplantation. However, even though this decrease in temperature considerably slows down cellular metabolism, it does not completely prevent the damage that is induced by oxygen deprivation, which is referred to as ischemia-reperfusion injury (IRI) (Blondeel et al., 2023).

The process and mechanism of SCS is divided into three steps:

1. Cooling: During the cooling process the organ is flushed with a cold preservation solution to remove blood and bring the temperature down. Oxygen demand is decreased because of this process, which slows down metabolic reactions within cells (Jing et al., 2018).
2. Storage: Following the cooling process, the organ is transferred to a sterile container that is filled with the same preservation solution, but also with ice. This ensures that the temperature remains consistently low during the entire preservation period (Jing et al., 2018).
3. Transport: To keep the container at the proper low temperature while it is being transported to the transplant center, the organ is often kept in an icebox or a specialized cooler (for example, an isothermal box) (Jing et al., 2018; Schlegel et al., 2018).

Table 1 - Advantages and limitations of SCS.

Static Cold Storage (SCS)		
Advantages	**Limitations**	**References**
Simplicity and Accessibility: SCS is a basic approach that does not required elaborated apparatus or equipment, which makes it accessible to most transplant centers.	**Limited Preservation Time:** Despite the extension of preservation time, SCS continues to place restrictions on the amount of time that an organ may be preserved. The average duration of liver preservation is 24h.	(Blondeel et al., 2023; Jing et al., 2018; Schlegel et al., 2018)
Effectiveness towards Standards Risk Organs: SCS is highly successful when it comes to preservation of organs that are not deemed to be high risk, such as those that come from brain-dead donors who are in good health.	**Risk of Ischemia-Reperfusion Injury:** SCS does not totally prevent IRI, which is a key cause of graft malfunction and failure after transplantation occurs.	
Cost-Effectiveness: In contrast to more sophisticated preservation methods like machine perfusion, SCS exhibits greater cost-effectiveness on account of its reduced operational expenses and minimal equipment requirements.	**Difficulty in Assessing Organ Viability:** Real-time evaluation of the organ's function is not possible with SCS, which can be especially difficult for high-risk organs, such as organs from ECDs. This presents a challenge when attempting to determine the viability of the organ.	
Slows Cellular Metabolism: SCS substantially reduces metabolic rate of the organ by means of chilling, consequently decreasing cellular demand for oxygen and mitigating potential harm for cells that may occur during transportation.	**Inadequate for High-Risk Organs:** There is a possibility that SCS might not provide appropriate protections for organs from ECDs or DCD, which can result in greater percentages of organs being discarded.	
Extended Preservation Times: SCS prolongs the duration of organ preservation outside of the body, which also benefits logistics such as transportation and recipient preparation.	**No Opportunity of Organ Repair:** Unlike dynamic preservation procedures such as machine perfusion, SCS does not provide the option to repair damage or enhance the status of organ prior to transplantation. This means that SCS does not help organs to recover from injury.	
Familiarity and Widespread Adoption: SCS is a method that has been around for a long time, is well known, and is widely utilized in most transplant programmes.	**Potential for Cold-Induced Damage:** The organ tissues may sustain cold-induced injury during extended periods of cold ischemia, which may impair their functionality following the transplant procedure.	

Although SCS has proven to be effective in the preservation of normal liver grafts, its limitations in preserving high-risk organs have sparked renewed interest in machine perfusion techniques (Czigany et al., 2020; Schlegel et al., 2018). The purpose of these techniques is to encourage the function of organs throughout the preservation process by providing oxygen and

nutrients. This might possibly prolong the viability of preserved organs beyond the constraints imposed by SCS (Czigany et al., 2020).

1.4.2. Dynamic Organ Preservation Techniques

The implementation of machine perfusion in organ transplantation represents a groundbreaking and progressive technological development that surpasses traditional approaches of organ preservation (Del Prete et al., 2022; Monbaliu & Brassil, 2010; Schlegel et al., 2015). In contrast to SCS, machine perfusion offers a dynamic circulation of a perfusion solution through the organs with numerous benefits, including: uninterrupted circulation and improved preservation of the microcirculation; uninterrupted delivery of nutrients and oxygen to meet the metabolic demands of the organ; elimination of metabolic waste products and toxins; ability to evaluate organ viability; enhanced clinical outcomes through improved rates of immediate graft function; extended preservation times without escalating preservation damage; and, delivery of cytoprotective and immunomodulating substances (Boteon et al., 2020; Jing et al., 2018; Monbaliu & Brassil, 2010; Patrono et al., 2023).

The implementation of machine perfusion techniques constitutes a critical turning point in the field of transplant medicine, presenting an increased capacity to enhance transplant outcomes and substantially broaden the pool of potential donors (Blondeel et al., 2023; Schlegel et al., 2018).

These techniques make use of a variety of temperature-specific methods, such as, Hypothermic Oxygenated Machine Perfusion (HOPE), Normothermic Machine Perfusion (NMP), and Subnormothermic Machine Perfusion (SNMP) (Del Prete et al., 2022; Jing et al., 2018; Monbaliu & Brassil, 2010; Schlegel et al., 2015).

1.4.2.1. Hypothermic Oxygenated Machine Perfusion (HOPE)

The Hypothermic Oxygenated Machine Perfusion (HOPE) technique represents a substantial departure from previous methods in the field of organ transplantation, particularly with regard to liver transplants (Figure 2). This technique operates under hypothermic conditions, within a

key temperature of 0ºC to 8ºC (Del Prete et al., 2022; Jing et al., 2018). The HOPE mechanism is based on the principle of sustaining oxidative energy generation by mitochondrial electron transport under hypothermic conditions. So, this capacity to produce and maintain ATP is critical for recharging the graft with tissue energy, thus increasing the likelihood of successful transplant (Jing et al., 2018).

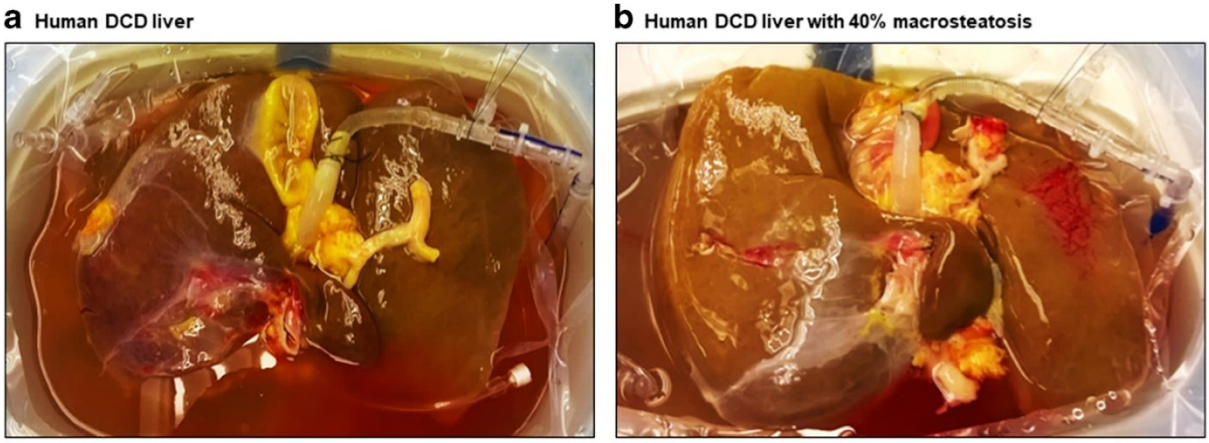

Figure 2 - Examples of hypothermic oxygenated perfusion of liver grafts prior to implantation. Retrieved from (Schlegel et al., 2018).

A critical component of HOPE is its capacity to restore ATP and counteract reverse electron transport, which enables HOPE to effectively prepare the liver to endure IRI, functioning as a protective mechanism against IRI (Figure 3) (Blondeel et al., 2023; Schlegel et al., 2018). This process includes a considerable period of mitochondrial reprogramming, which allows the organ to switch from an ischemic condition to a completely ATP-load state, within 1-2 hours of cold oxygenated perfusion (Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021). Through the process of reintroducing oxygen under hypothermic conditions, mitochondria can efficiently recover ATP levels and metabolize stored succinate and NADH, hence reducing the inflammatory signaling that is generated by the production of ROS within the context of normothermic reperfusion (Del Prete et al., 2022; Guibert et al., 2011a). This type of reprogramming is crucial to minimize inflammation and oxidative damage in mitochondria, both of which are essential for improving outcomes after a transplant (Del Prete et al., 2022; Panconesi, Flores Carvalho, et al., 2021; Schlegel et al., 2018). Throughout the preservation period, the liver is exceptionally vulnerable to metabolic disturbances (Schlegel et al., 2015). As an essential component of hepatic metabolism, oxygen assumes a critical function since it has a direct effect on mitochondrial function. The oxygenation of mitochondria at

hypothermic temperatures is an essential component of the HOPE approach, which is used to preserve the mitochondria integrity and function (Guibert et al., 2011a; Karangwa et al., 2020; Schlegel et al., 2015). This method of reoxygenating the liver under regulated settings makes it possible to restore mitochondrial functions in a gradual and steady manner, so that sudden alterations are unlikely to occur, which can, consequently, lead to oxidative stress and cellular damage (Guibert et al., 2011a; Karangwa et al., 2020; Schlegel et al., 2015). Also, by effectively controlling oxygen levels throughout the perfusion, the metabolic demands of the liver are adequately fulfilled, and the potential harm is reduced, both of which are crucial prior to transplantation (Guibert et al., 2011a; Karangwa et al., 2020; Schlegel et al., 2015).

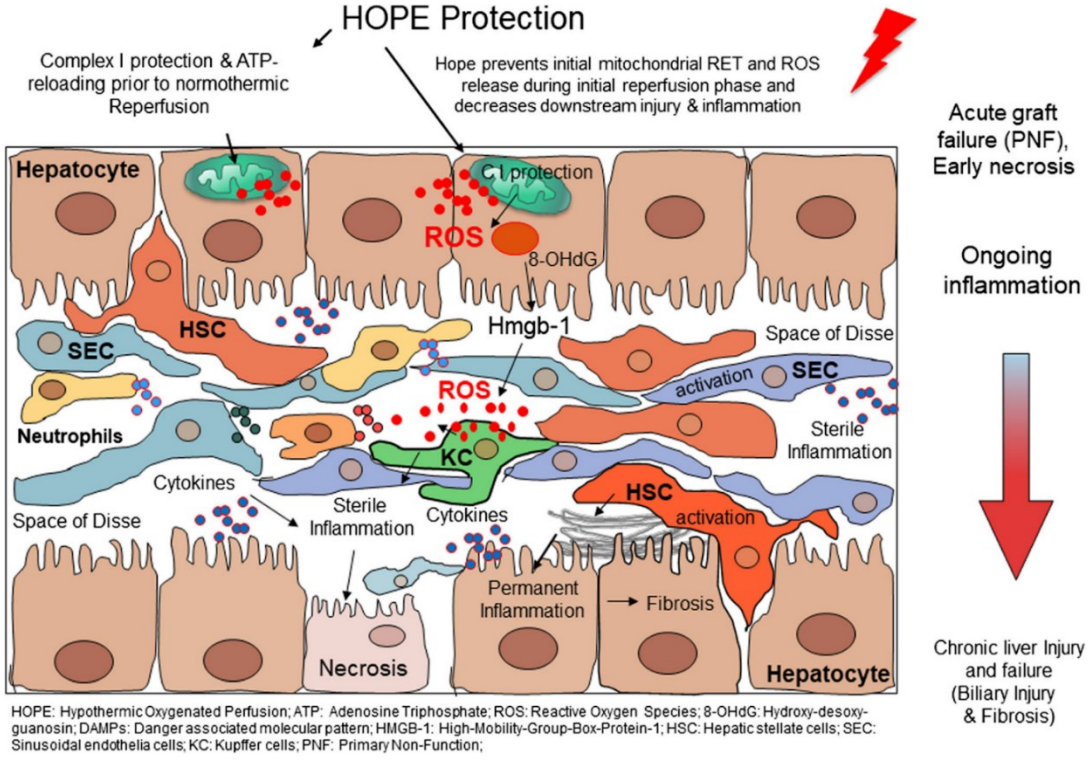

Figure 3 - Mechanism of injury following ischemia/reperfusion and protection through hypothermic machine perfusion approaches. Retrieved from (Schlegel et al., 2018).

Clinical trials have demonstrated that HOPE exhibits enhanced graft survival and decreased post-transplant complications in comparison to the conventional SCS (Del Prete et al., 2022; Monbaliu & Brassil, 2010). Also, as a demonstration of the effectiveness of HOPE, recipients of livers that undergone HOPE procedure had reduced post-operative indicators of IRI, shorter hospitals stays and other post-transplant complications (Del Prete et al., 2022; Patrono et al., 2023). These advantages are highlighted in a number of clinical studies, which underscores the

increasing attention and potential of this technique within the wider domain of organ transplantation (Figure 4) (Del Prete et al., 2022; Monbaliu & Brassil, 2010; Patrono et al., 2023).

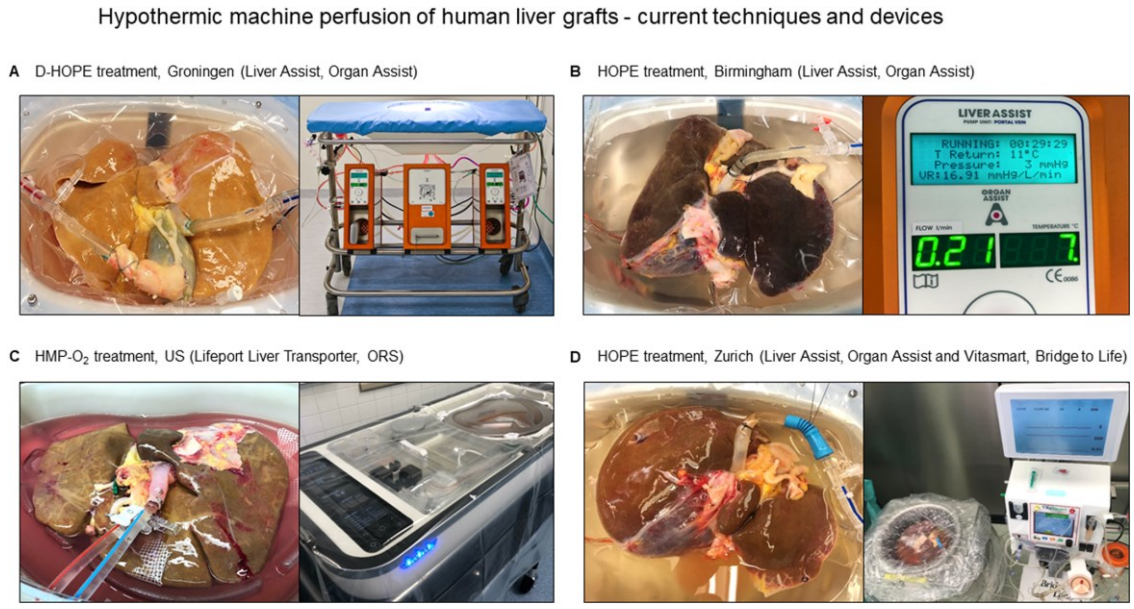

Figure 4 - Hypothermic machine perfusion of human liver grafts – current techniques and devices. Retrieved from (Karangwa et al., 2020).

From an economic and logistical standpoint, even though HOPE is more expensive than SCS, it is less expensive than other preservation techniques, being a straightforward and cost-effective strategy that facilitates the utilization of organs that otherwise would be considered unsuitable for transplantation, while minimizing post-transplant complications (Del Prete et al., 2022). Given its economic viability and compatibility with conventional organ procurement, HOPE emerges as a pragmatic alternative within the realm of organ preservation methodologies (Del Prete et al., 2022; Patrono et al., 2023).

Nevertheless, the execution of HOPE is accompanied by technical obstacles and specific areas that need more investigation. One of the continuing areas of research is figuring out the ideal amounts of oxygenation and perfusion pressures (Guibert et al., 2011a; Schlegel et al., 2015). Because the liver tissues continue to consume oxygen during HOPE, the oxygenation debate is substantial. Although the liver is in a hypothermic state (0ºC-8ºC), oxygen is still necessary for metabolic processes that are reduced. This raises questions about the ideal level of oxygenation required to sustain the metabolic demands of the liver while preventing the development of oxidative stress (Guibert et al., 2011a; Schlegel et al., 2015). A proper oxygenation balance is of the utmost importance, as it has a direct impact on the condition and viability of the liver

prior to transplantation. Considering this, adequate oxygenation has the potential to improve liver preservation, mitigate IRI, and possibly improve liver transplant success rates (Guibert et al., 2011a; Schlegel et al., 2015).

In addition to this, the duration and pressure are also critical factors to consider during HOPE. Elevated perfusion pressures are correlated with an increased likelihood of endothelial damage, emphasizing the importance of sustaining low perfusion pressures to reduce shear stress on the endothelium (Schlegel et al., 2015, 2018).

Considering all of the above reasons, HOPE is considered a promising advancement in the field of organ preservation, particularly for liver transplantation. Because of its capacity to maintain mitochondrial function, decrease IRI, and enhance post-operative results, it is an intriguing method in the field of transplantation (Blondeel et al., 2023; Czigany et al., 2020; Del Prete et al., 2022; Schlegel et al., 2015).

1.4.2.2. Normothermic Machine Perfusion (NMP)

The Normothermic Machine Perfusion (NMP) technology offers a dynamic preservation method that closely replicates physiological settings (Boteon et al., 2020; Jing et al., 2018). This approach marks a substantial paradigm change in organ transplantation. NMP, which functions at body temperature (35ºC-38ºC), is of utmost importance in maintaining the viability and metabolic activity of donor organs (Del Prete et al., 2022). In NMP, the perfusion fluid contains either donor blood or blood substitute enriched with essential nutrients and oxygen (Boteon et al., 2020; Jing et al., 2018).

In contrast to other preservation techniques, such as HOPE, in which the perfusion fluid usually consists of a cold, oxygenated, and acellular solution, the NMP distinguished by the use of blood or blood-like solutions (Jing et al., 2018; Monbaliu & Brassil, 2010).

This technique fundamentally seeks to mimic *in vivo* settings, aiming at minimizing damages associated with organ preservation, enabling in real-time evaluate organs functions, and activate processes for tissue healing (Boteon et al., 2020; van Beekum et al., 2021). NMP serves as an intermediary through which organ-specific therapeutics can be administrated, regenerative

pathways can be stimulated, and apoptosis and immune responses can be regulated. These processes are critical for the restoration of organ metabolism and for the improvement of organ quality prior transplantation (Boteon et al., 2020; Patrono et al., 2023; van Beekum et al., 2021). Additionally, NMP inhibits further ATP depletion, restricts the accumulation of metabolic waste products, and enables continuous monitoring and evaluation of organ functionality by simulating normal physiological conditions (Boteon et al., 2020; van Beekum et al., 2021).

NMP has exhibited potential in clinical settings by offering advantages over conventional preservation methods, such as SCS. In fact, these benefits include decreased levels of post-transplant AST and extended preservation periods that do not result in increased preservation damages. Additionally, NMP has shown to decrease post-reperfusion syndrome and early allograft dysfunction (Boteon et al., 2020; Patrono et al., 2023).

In summary, NMP is a revolutionary method in organ transplantation that preserves and assess organ viability under near-physiological conditions. Regardless of its technical complexity and resource demands, further investigation and clinical trials are anticipated to improve its implementation and expand its scope of use within clinical environments (Boteon et al., 2020; Patrono et al., 2023; van Beekum et al., 2021).

1.4.2.3. Subnormothermic Machine Perfusion (SNMP)

In the field of organ transplantation, Subnormothermic Machine Perfusion (SNMP) is becoming more popular as a novel strategy that provides a balance between HOPE and NMP. Considering this, SNMP attempts to combine the metabolic activity of normothermic conditions with the decreased metabolic demand of hypothermia, operating within the temperature range of 24ºC-34ºC (Del Prete et al., 2022; Jing et al., 2018). This perfusion technique exhibits a promising potential in extending the viability of organs that are intended for transplantation (Monbaliu & Brassil, 2010).

Comparing to NMP, SNMP has lower metabolic requirements, which allows for some degree of organ function evaluation and maintenance. This is a common advantage of all machine perfusion techniques when compared to SCS (Monbaliu & Brassil, 2010; Schlegel et al., 2015). Furthermore, since this technique combines aspects from HOPE and NMP, it is possible to state that SNMP solves some of the drawbacks that are associated with these perfusion techniques

(Bruinsma et al., 2014; Monbaliu & Brassil, 2010; Schlegel et al., 2015). This is particularly pertinent in the light of the concerns about the high temperature increase that NMP causes in livers that are damaged, ischemic, or cold-stored (Bruinsma et al., 2014). By providing a more incremental rewarming trajectory, SNMP may mitigate the stress that is commonly associated with the rapid increase in temperature normalization and metabolic demand (Bruinsma et al., 2014). Furthermore, the capacity of SNMP to work well at lower temperatures without the need of oxygen carriers simplifies the system, possibly accelerating its clinical application (Bruinsma et al., 2014).

SNMP represents an extraordinary development in the field of organ transplantation, as it provides a unique balance between hypothermia's reduced metabolic demands and normothermia's active state (Guibert et al., 2011a; Jing et al., 2018; Schlegel et al., 2015). However, SNMP lacks comprehensive clinical data as compared to proven procedures like HOPE and NMP, which suggest that the practical application of

SNMP deserves more extensive investigation and clinical testing (Bruinsma et al., 2014; Monbaliu & Brassil, 2010; Schlegel et al., 2015).

1.4.2.4. Comparison Between the Different Machine Perfusion Techniques

From the information mentioned before, three methods stand out in the domain of dynamic preservation techniques for organ transplantation: HOPE, NMP and SNMP.
By regulating mitochondrial function and preventing IRI at low temperatures (0-8ºC), HOPE is especially advantageous for organs several organs, like liver, kidneys, and hearts and lungs (Del Prete et al., 2022; Guibert et al., 2011a; Jing et al., 2018; Schlegel et al., 2015). By operating at body temperatures (35-38ºC) NMP replicates the physiological conditions, preserving the viability of the organ and enabling the real-time evaluation and restoration of the donor organ (Boteon et al., 2020; Jing et al., 2018; Patrono et al., 2023). Finally, SNMP serves as an intermediary between HOPE and NMP by functioning at subnormothermic temperatures (20-34ºC) with the objective of mitigating metabolic stress while maintaining a portion of the vital metabolic activity (Monbaliu & Brassil, 2010; Schlegel et al., 2015).

The graph below (Figure 5) illustrates the metabolic rate of an organ at various temperatures during the different preservation methods mentioned above.

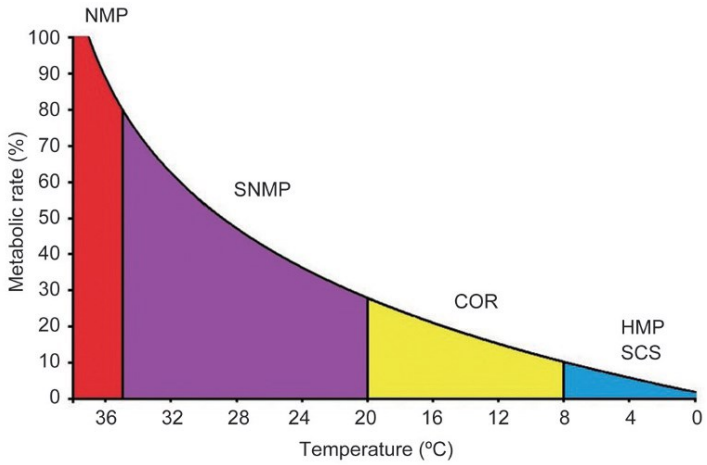

Figure 5 - Metabolic rate reduces with a decrease in temperature in humans. Retrieved from (Jing et al., 2018).

Therefore, considering the advantages and disadvantages inherent to each dynamic perfusion technique, their use will be determined by the organ to be preserved and transplanted. In this work, HOPE was of particular interest due to its notable advantages (Blondeel et al., 2023; Schlegel et al., 2018). The key factor is the impact of HOPE on mitochondrial activity. In hypothermic conditions, its efficiently maintains and improves mitochondrial function, an essential requirement for sustaining organ viability throughout the preservation process (Del Prete et al., 2022; Jing et al., 2018). This feature of HOPE is especially beneficial in comparison to NMP and SNMP, as the reduced metabolic demand cause by HOPE's lower temperature range effectively mitigates cellular stress and metabolic imbalances (Guibert et al., 2011a; Karangwa et al., 2020; Schlegel et al., 2015). Moreover, the operational simplicity of HOPE enhances its potential for extensive implementation in clinical settings. The process is considerably simplified due to the technique's reliance on lower temperatures, which eliminates the necessity for complex nutrient solutions and demanding metabolic controls that are inherent in NMP and SNMP (Del Prete et al., 2022; Guibert et al., 2011a; Jing et al., 2018; Schlegel et al., 2015). Furthermore, cost factors are of the utmost importance, since HOPE is more economically feasible overtime as a result of its reduced operational expenses and absence of costly supporting components, rendering it a more economical long-term solution (Del Prete et al., 2022; Guibert et al., 2011a; Jing et al., 2018; Schlegel et al., 2015).

1.4.3. Preservation Solutions

Preservation solutions are of the utmost importance in organ preservation. These solutions serve as supportive fluids, specifically formulated to address the intricate requirements of organs situated externally to the human body. They give protection against cellular damage, maintain the pH balance, and provide vital nutrients (Guibert et al., 2011a; Jing et al., 2018; Patrono et al., 2023). The composition of these solutions normally consists of a well-balanced combination of electrolytes and impermeant agents, and in certain instances, additional chemicals that are designed to help counteract oxidative stress and provide metabolic substrates. The advantages of each formulation are distinct and customized to suit the preservation needs of each organ type (Guibert et al., 2011a; Patrono et al., 2023). The persistent dedication of the field to continuous research and development serves to emphasize the critical importance of these solutions in transplant medicine by demonstrating an ongoing effort to improve organ preservation outcomes (Jing et al., 2018).

Pioneers in this field, the Collins and Euro-Collins solution were widely applied to a variety of organs owing their capacity to protected against prolonged cold ischemia (Guibert et al., 2011a; Jing et al., 2018). The University of Wisconsin (UW) Solution was developed in the mid-1980s, and became the gold standard for abdominal organs, especially liver grafts, due to its well-balanced composition and ability to preserve organ integrity for a duration up to 24 hours (Guibert et al., 2011a; Jing et al., 2018). In contrast to UW, the Histidine-Tryptophan-Ketoglutarate (HTK)/Custodiol Solution, which was originally developed to treat cardioplegia, became well-known for its efficient organ chilling capabilities and decreased viscosity (Guibert et al., 2011a; Jing et al., 2018; Patrono et al., 2023). The more recent Celsior Solution, an extracellular-type solution, and the IGL-1 solution, developed for multi-organ preservation, both integrate components from prior solutions and introduce novel substances (Guibert et al., 2011a; Patrono et al., 2023).

The selection of these solutions is based on their distinct compositions, which accommodate the physiological requirements of liver transplants. These solutions provide electrolyte balance, ischemic damage protection, cellular metabolism support, and assistance throughout the critical preservation period (Guibert et al., 2011a; Patrono et al., 2023).

The detailed composition of each of the above preservation solutions discussed are present in Table 7 in the Section I of the Appendix.

1.5 VIABILITY ASSESSMENT TECHNIQUES

Prior to organ transplantation, it is of the utmost importance to evaluate the viability of organs, particularly in the case of ECDs, who have a greater possibility of post-transplant complications (Panconesi, Carvalho, et al., 2021). Over the course of the last decades, there has been a need for new evaluation methods that can make the organ evaluation more objective contributing to a reduction of the risks and enhancing transplant outcomes (Jing et al., 2018). In light of this, it is imperative to search for a specific biomarker that may offer a prediction value of viability throughout the whole process of transplantation (Jing et al., 2018). In fact, the use of such biomarkers would significantly improve the objectivity and accuracy of evaluations, representing a considerable advancement in comparison to the conventional methods, which are often subjective (Jing et al., 2018; Panconesi, Carvalho, et al., 2021). Furthermore, following the biomarkers evaluation will allow to optimize donor-recipient matching and reduce organ waste (Wang et al., 2020). At the moment, there are no scoring systems or biomarker that has been properly validated for assessing liver viability that is being used in clinical settings to simplify the decision-making process for transplant teams (Wang et al., 2020). Considering this, recent research has investigated Flavin Mononucleotide (FMN) as a potential biomarker for detecting organ viability in liver transplants, in the context of HOPE (Karangwa et al., 2020; Panconesi, Flores Carvalho, et al., 2021).

Traditionally, the evaluation of a donor's liver quality consists of a mix of surgical expertise and a few predictors, including laboratory testing, liver biopsy, and past medical records (Table 2). During the procurement surgery, organs are evaluated in terms of their shape, size, color, flush, and vessel quality, and the recipient center is promptly notified of the findings, and, in some cases, additional tests are performed (Panconesi, Carvalho, et al., 2021). Other analyses include routine blood gas or biochemical analysis to the cold storage solutions or machine perfusates, including to lactate, liver transaminases (AST, ALT) or pH measures (Del Prete et al., 2022; Panconesi, Carvalho, et al., 2021). However, most of these markers are not specific and have a limited predictive value (Panconesi, Carvalho, et al., 2021). As example, the AST and ALT levels can be influenced by other factors such as hemolysis, which may result in inaccurate evaluations of the viability of the organ (Panconesi, Carvalho, et al., 2021). In a similar way, despite their prominence in viability evaluation, lactate levels frequently fail to differentiate viable livers from non-viable livers (Panconesi, Carvalho, et al., 2021).

Therefore, the application of spectroscopy to measure FMN concentrations has become a significant advancement, offering a more accurate and instantaneous approach of assessment. This novel methodology represents a substantial milestone in the field of transplant medicine, presenting an opportunity to fundamentally transform the evaluation procedure through the provision of unbiased, measurable information to help decision in organ transplantation (Eden et al., 2023; Huwyler et al., 2023; Karangwa et al., 2020; Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021).

Table 2 - Overview of different techniques available to assess the donor liver quality during donation, preservation, and transplantation. Retrieved from (Panconesi, Carvalho, et al., 2021).

Detection Method	Material	Parameters in Clinical Use	Time Needed for Assessment	Advantages	Disadvantages
Macroscopic Assessment	Entire liver	Size, Perfusion quality, Stetatosis, Fibrosis, Vessel quality, Injuries	Minutes	Routine, Rapid, Non-invasive, Cheap	No information on function, Imprecise, Assessor dependent
Ultrasound	Entire liver	Size, Level of steatosis, Lesions	30 min	Easy, Assessment of liver parenchyma, Rapid, Cheap	Assessor dependent, No information on function
Fibroscan	Entire liver	Level of fibrosis	30 min	Non-invasive, Simple, Rapid, Reproducible, Non-operator dependent	No information on function, Additional costs
Histology	Entire liver	Level of macro and microsteatosis, Fribrosis, Inflammation	1-2h	Histological evidence of quality provides criteria to exclude organ transplantation (e.g, Fibrosis)	Invasive, Variability on interpretation; Biopsy covers only small part of organ, No information on function
Heamodyn-amics during Perfusion	Entire liver	HA & PV perfusion flow (pressure)	Continuous	Real-Time	Not specific for cell type
Blood gas analysis	Perfusate, Effluate, Bile	pO2, pCO2, Lactate, Na, K, pH, Glucose	5- 15 min	Non-invasive, Any type of perfusion, Multiple parameters, Indirect cholangiocyte assessment, Different time points	Timing, Different parameters, Not specific for a certain cell time
Biochemical analysis	Perfusate, Effluate, Bile	AST, ALT, LDH, HCO3-, ALP	5-15 min	Non-invasive, Indirect cholangiocyte assessment, Different time points	Not specific for a certain cell type, No functional assessment, No reliable prediction of outcomes after transplantation
Spectroscopy	Perfusate, Effluate	FMN, NADH	5-15 min	Easy, Quick, Chep, Reliable prediction of graft function, Covers entire organ, Any time of machine perfusion	No discrimination between different cell types
Metabolomic/ Proteomics/ Genomics	Liver tissue, Perfusate, Effluate, Bile	Various molecules from all cellular and sub-celullar compound	Days/weeks	Multiple parameters, Can be performed in any material (tissue, perfusate, bile)	Requires long time, Expensive, Not for specific for a certain cell type

The flowchart below (Figure 6) details the comprehensive process of liver transplantation from donor assessment to transplantation, showing the difference stages in the process.

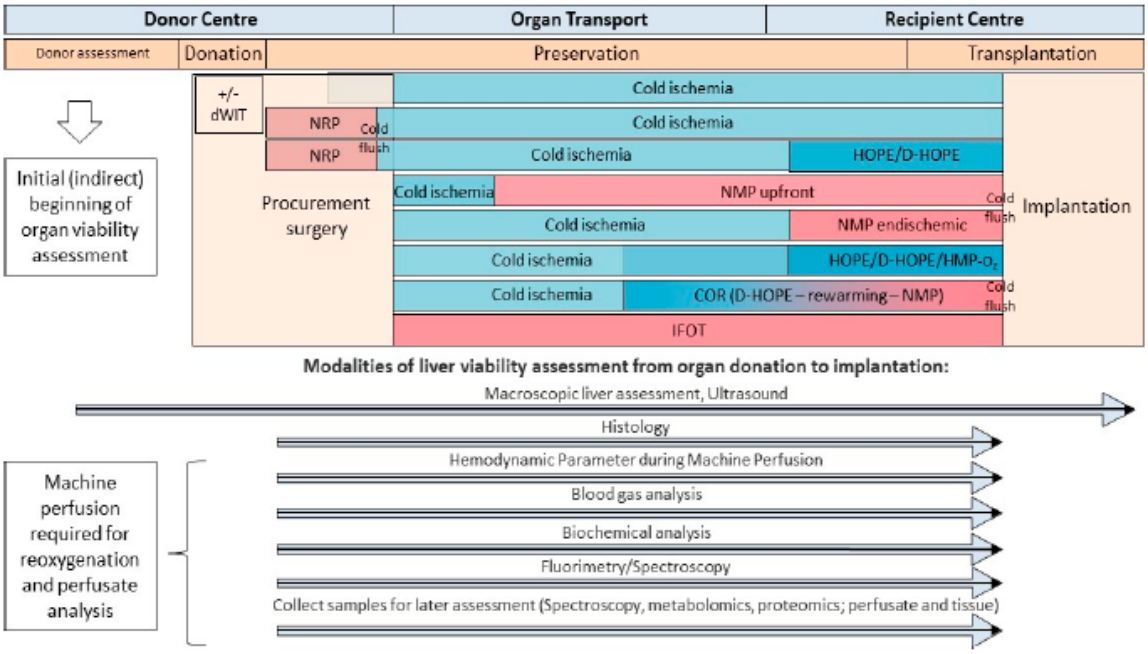

Figure 6 - Timeline and modalities to assess viability from organ donation, during preservation, and transplantation. Retrieved from (Panconesi, Carvalho, et al., 2021).

1.5.1. Flavin Mononucleotide (FMN) as a Predictive Biomarker

FMN, which is a marker of mitochondrial complex I injury, correlates with graft survival, post-transplant complications, and ATP degradation (Blondeel et al., 2023). A clear association exists between the release of this molecule from the liver during HOPE, and not only the damage of the liver but also the functional results that occurs after liver donation (Blondeel et al., 2023; Karangwa et al., 2020; Panconesi, Flores Carvalho, et al., 2021). As a consequence, distinct FMN threshold values were established and implemented in the deliberative phases of HOPE (Eden et al., 2023; Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021). When employing FMN as a biomarker in liver transplantation, the method of measurement and scheduling of analysis are crucial components. Quantification of FMN levels in the perfusate is accomplished using spectroscopy, a method that enables the accurate identification of this biomolecule. The samples for this analysis are collected during the HOPE procedure (Del Prete et al., 2022; Karangwa et al., 2020; Panconesi, Flores Carvalho, et al.,

2021). In the HOPE technique, the most important time periods for collecting the samples and conducting analysis are at 30 and 60 minutes into the procedure. The time limits are crucial because they provide data that is required for making well-informed decisions regarding the liver's suitability for transplantation (Eden et al., 2023; Huwyler et al., 2023; Panconesi, Carvalho, et al., 2021).

The mechanism of FMN as a viability marker revolves around mitochondrial function and injury (Figure 7). So, for cells to generate energy efficiently mitochondrial function, specifically within complex I is indispensable (Blondeel et al., 2023). During ischemia, which is, as it was mentioned before, the absence of oxygen flow to the organ after its removal from the donor, the mitochondria are unable to carry out aerobic respiration in an effective manner (Eden et al., 2023; Panconesi, Carvalho, et al., 2021). As a result, cells switch to anaerobic respiration, which is defined as an energy-producing mechanism that does not require oxygen, but produces less energy (Eden et al., 2023; Panconesi, Carvalho, et al., 2021). This process results in the buildup of electron donors in the mitochondria, such as Nicotinamide Adenine Dinucleotide (Hydrogen) (NADH) and succinate (Del Prete et al., 2022; Eden et al., 2023; Huwyler et al., 2023). This accumulation implies a disruption in the electron transport chain normal function (Wang et al., 2020). As a response to this disruption and the lack of oxygen, cellular metabolism shifts to anaerobic glycolysis, which generates lactate (Panconesi, Carvalho, et al., 2021). Although this transition serves as a compensatory mechanism for energy production, it also signifies ischemic stress in the cells. Upon the reintroduction of oxygen (reperfusion) during HOPE procedure, complex II in the mitochondria rapidly oxidizes the accumulated succinate (Huwyler et al., 2023; Wang et al., 2020). As a consequence of this abrupt oxidative effect, the mitochondrial electron transport system may become unbalanced, leading to the biochemical process of reverse electron transfer at mitochondrial complex I (Eden et al., 2023; Wang et al., 2020). This irregular flow of electrons has the potential to contribute to the production of $FMNH_2$ (reduced flavin mononucleotide) as well as ROS (Del Prete et al., 2022; Panconesi, Carvalho, et al., 2021). When the mitochondrial complex I is under stress, $FMNH_2$ can be released into the mitochondrial matrix. When this molecule is exposed to the high oxygen environment present during reperfusion, it gets oxidized into FMN (Panconesi, Carvalho, et al., 2021). Subsequently, the FMN is released from the mitochondria into the cell, and eventually into the surrounding perfusate (Eden et al., 2023; Huwyler et al., 2023).

An essential element of FMN's biomarker functionality is its inverse correlation with perfusate concentrations (Blondeel et al., 2023; Wang et al., 2020). Thus, low levels of FMN within the mitochondria of the liver are correlated with high levels of FMN in the perfusate. FMN is released from the mitochondria into the perfusate when mitochondrial function is disrupted, indicating considerable damages to the organelle and a compromise of the liver for transplantation (Eden et al., 2023; Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021; Wang et al., 2020). On the other hand, low levels of FMN in the perfusate indicate that the liver is retaining the FMN, which serves as an indication of mitochondrial activity and liver suitability for transplantation (Eden et al., 2023; Huwyler et al., 2023; Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021). Overall, this correlation between the levels of FMN in perfusate and mitochondrial activity at the liver provides an essential insight into the health state of the organ, which in turn enables a more precise evaluation of the organ's potential for transplantation (Blondeel et al., 2023; Eden et al., 2023).

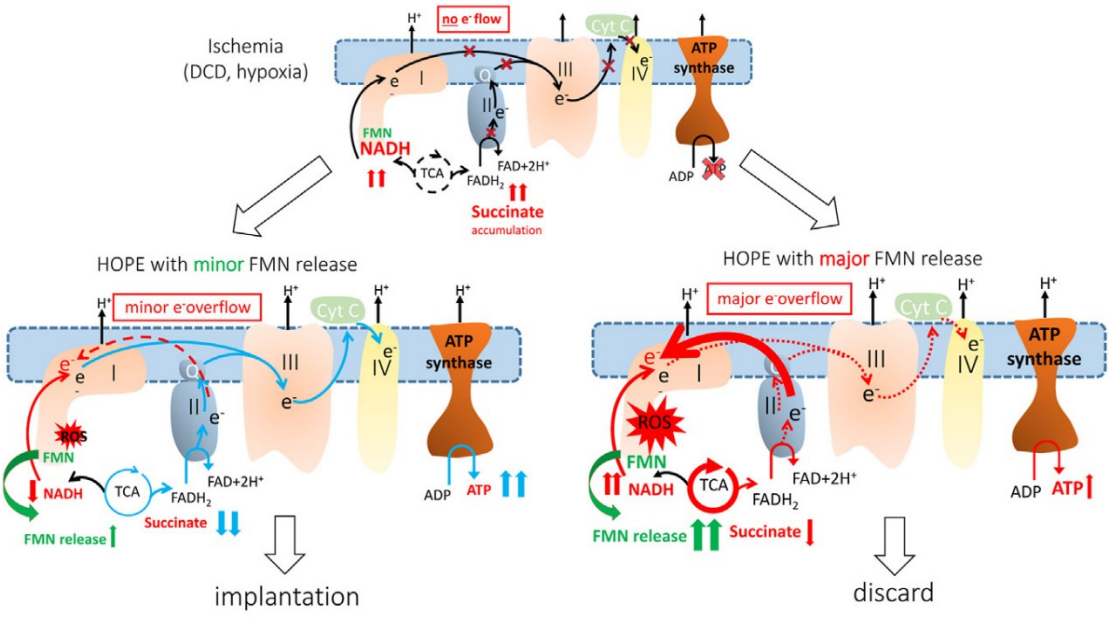

Figure 7 - Mechanism of mitochondrial injury and assessment of mitochondrial function during HOPE. Retrieved from (Eden et al., 2023).

Notable among the contributions of the Zurich group, which is one of the pioneers in the machine and have been studying for the past years several aspects of machine perfusion, is the development of a spectrum of FMN values that serve as an indicator of liver viability (Figure 8)

(Boteon et al., 2020; Del Prete et al., 2022; Eden et al., 2023; Patrono et al., 2023). Due to the minimal level of mitochondrial damage, livers with FMN ≤ 6000 A.U (which approximately corresponds to a FMN perfusate concentration of 0.03 µg/mL) within the initial hour of HOPE were taken into consideration for transplantation (Eden et al., 2023). On the other hand, livers exhibiting levels of FMN ≥ 6000 A.U (which means perfusate FMN concentrations ≥ 0.03 µg/mL) were discarded (Eden et al., 2023). The explicit delineation of FMN values for the purpose of decision-making underscores the accuracy and dependability of FMN as a liver transplantation biomarker (Eden et al., 2023). The presented evidence supports the utility of FMN as a prognostic biomarker for liver viability and favourable outcomes in transplantation.

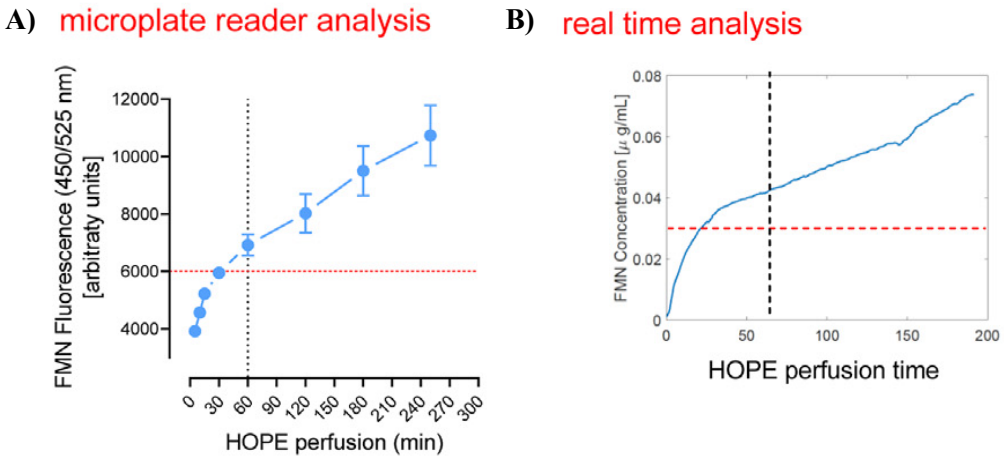

Figure 8 - Analysis of FMN levels during HOPE perfusion. Retrieved from (Eden et al., 2023).

A paradigm shift in transplant medicine has occurred with the incorporation of FMN analysis into the liver viability evaluation procedure. This gives a more objective, quantitative, and real-time measure of the health of the liver, especially regarding mitochondrial function and damage assessment (Boteon et al., 2020; Karangwa et al., 2020; Panconesi, Carvalho, et al., 2021; Panconesi, Flores Carvalho, et al., 2021).

2 OBJECTIVES

The work developed under the present master's thesis, conducted as part of a curricular internship at Centro Hospitalar Universitário de Santo António (CHUdSA), aims at expanding knowledge on liver perfusion, using the Hypothermic Oxygenated Machine Perfusion (HOPE) technique and its impact on liver transplantation.

The comprehensive review in Section 1, summarizing the state-of-the-art, established solid theoretical foundation in the field of organ perfusion, supporting the development of two specific objectives of the thesis:

- To define the operational parameters for the HOPE equipment to improve the health and viability of livers, particularly those from Expanded Criteria Donors (ECDs).

- To assess liver viability using the Flavin Mononucleotide (FMN) biomarker as a route to increase the donor pool while improving transplant outcomes and therefore, the emerging practices in organ transplantation.

The first objective has been supported by the CHUdSA, while the second objective, was developed at Escola Superior de Biotecnologia da Universidade Católica Portuguesa (ESB-UCP). A critical aspect of this study is tackling the hospital's present limitation in conducting real-time liver viability assessments due to the lack of essential equipment. For that reason, a retrospective method was implemented, assessing the liver samples after transplantation to determine the efficacy of FMN as a predictive biomarker. Thus, this study attempts to determine if this biomarker can offer valid forecasts on its own or if it should be combined with traditional evaluation methods.

This thesis addresses the barriers in both theoretical and clinical research in the field of liver transplantation. Ultimately, the purpose of this thesis is not only to broaden the theoretical understanding of liver organ perfusion and viability assessment, but also to translate this knowledge into practical applications that can be used in hospital settings, thereby improving patients care and the success of liver transplants.

3 EXPERIMENTAL SECTION

This study required the development of two comprehensive protocols focusing on the topic of organ perfusion. The first protocol specifies the methods and criteria for using Hypothermic Oxygenated Machine Perfusion (HOPE, VitaSmart, Bridge to Life, USA) technology. This protocol not only describes the HOPE system's operational requirements, but it also specifies the crucial parameters for patient monitoring after the transplantation.

The second protocol concentrates on determining the viability of the liver and it is divided into two phases. The first requires the systematic collection of perfusion samples, for which certain criteria have been defined. The second phase focuses on laboratory analyses, which include the preparation of the samples to measure the levels of the liver biomarker, flavin mononucleotide (FMN). These parameters are critical in determining if a liver is suitable or not for transplantation.

Both protocols comply with the standards and practices established by the Centro Hospitalar Universitário de Santo António (CHUdSA) and are structured into several key sections, namely: Objective/Framework, Scope, Definitions/Evidence, Responsibility, Methodology, and Bibliography. The entire procedures, as authorized by the hospital, are supplied in the Appendix Section II for thorough reference.

For such, this study comprised a cohort of 24 livers obtained from ECDs, all of them were subject to the HOPE procedure. Subsequently, a subset of 6 livers were selected to undergo a comprehensive viability evaluation via the quantification of FMN concentrations in perfusion fluid samples. This evaluation provides a predictive assessment of liver viability before transplantation, representing a cutting-edge advancement in transplant medicine.

3.1 PART 1: HYPOTHERMIC OXYGENATED MACHINE PERFUSION (HOPE) PROTOCOL DEVELOPMENT AND EVALUATION

1. Donor Acceptance Criteria

When the liver is retrieved from the donor, it is carefully preserved on ice (hypothermic temperatures) to maintain its structural and functional integrity until it is ready to be subject to the HOPE procedure. A meticulously coordinated sequence of events takes place in the operating room (OR), where the transplant team prepares for the procedure as soon as a donor liver arrives to CHUdSA. Before proceeding with the liver transplant, the transplant surgeons perform a comprehensive evaluation to ascertain the liver suitability for donation.

In the presented study 24 livers from several hospitals were submitted to the HOPE procedure, and all of them were transplanted. All livers were acquired from donors with expanded criteria for the HOPE procedure. The criteria for these donors were adapted from recommendations made during the Paris Consensus Meeting on ECDs for liver transplantation.

The defined criteria include:

- Donor age of 65 years or older;
- Body Mass Index (BMI) of 30 kg/m2 or higher;
- Total bilirubin levels of 2,0 mg/dL or higher;
- Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) levels surpassing 500 U/L;
- Inclusion of donors with non-beating heart;
- Cold ischemia time surpassing 10 hours;
- Administration of two or more vasopressors at any given point;
- A history of mechanical ventilation extending beyond 5 days;
- Presence of steatosis in more than 30% of the liver tissue.

The collected livers were packaged and transported to CHUdSA following standard procedures.

2. Use of VitaSmart (Bridge to Life)

After conducting the critical evaluation of the liver's suitability for transplantation, the donor liver is prepared which is a process that involves meticulous surgical techniques to ensure the organ is ready for perfusion. Simultaneously, the HOPE equipment is activated and carefully calibrated to the specific requirements of the perfusion process. As shown in the figure below (Figure 9) the liver is connected to the HOPE system, following the configuration of the machine and confirmation that it is prepared, and then the perfusion starts.

The parameters set for the functioning of the perfusion machine are as follows:

- A minimum perfusion duration of 2 hours;
- A temperature range kept between 4 ºC to 12 ºC;
- Perfusate volume ranging between 2 L to 3 L, adjusted according to graft volume;
- Flow rate not exceeding 250 mL/min;
- Pressure maintained at maximum of 3 mmHg.

Figure 9 - VitaSmart Machine for HOPE procedure.

3. Patient Monitoring

In the critical phase following liver transplantation, meticulous monitoring of the patient's clinical parameters is crucial to ensure successful graft function and to promptly identify potential complications. The 24 transplanted patients were monitored according to the followed key aspects:

- Onset of reperfusion syndrome following graft implantation;
- Regular analysis of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Total Bilirubin, Lactate, and Creatinine on post-transplant 1,3, and 7, as these biomarkers provide crucial insights into liver function;
- Criteria to detect both early and delayed graft dysfunctions;
- Vascular complications, particularly hepatic artery thrombosis;
- Duration of both the overall hospital stay and time spent in intensive care;
- Late-stage biliary complications;
- Instances of retransplantation;
- Overall graft survival rate.

These parameters are standard criteria pre-defined by CHUdSA, however they are not exclusive and may vary according to the entity responsible for the transplant.

3.2 PART 2: VIABILITY ASSESSMENT OF THE LIVER POST-HOPE PROCEDURE

1. Sample Collection at CHUdSA

Among the cohort of 24 livers that underwent HOPE procedure, it is particularly significant to mention that perfusion fluid samples were collect in only 6 cases to perform a more focused viability assessment.

The perfusion fluid samples were collected at CHUdSA during the 6 HOPE procedures, at the following time-points:

- T0: 0 minutes - Beginning of HOPE (baseline value);
- T1: 5 minutes post-HOPE initiation;
- T2: 10 minutes post-HOPE initiation;

- T3: 15 minutes post-HOPE initiation;
- T4: 30 minutes post-HOPE initiation;
- T5: 60 minutes post-HOPE initiation;
- T6: 90 minutes post-HOPE initiation;
- T7: 120 minutes post-HOPE initiation;

For each of these time-points, two samples were collected, resulting in a total of 16 samples. Samples were collected into 1 mL Eppendorf tubes and kept at -20 ºC, protected from light, at Escola Superior de Biotecnologia da Universidade Católica Portuguesa.

2. Sample Analysis at ESB-UCP

2.1. FMN Quantification

This pioneering and groundbreaking investigation utilized retrospective analysis to quantify FMN subsequent to the transplantation procedures, as a result of equipment constraints at the hospital at the present time. Initiating a pilot study with the objective of establishing a correlation between FMN levels and patient outcomes and comparing these results to prior research in the field was the motivation for this strategy. One of the main objectives of this study is the validation of FMN quantification as a reliable method that could potentially be integrated into the real-time workflow of HOPE procedure in the OR. In order to establish FMN as a universally recognised predictive biomarker of liver viability, this effort must ultimately succeed. Real-time evaluation of FMN levels throughout the HOPE process could provide an accurate assessment of liver viability prior to transplantation, potentially increasing patient outcomes and streamlining the decision-making process.

FMN quantification was performed to assess liver's viability after the 6 HOPE's procedures, according to previous published works (Eden et al., 2023)

Samples were thawed at room temperature and kept on ice during analysis. For FMN quantification, 150 µL of 0.9% (w/v) of sodium chloride solution (NaCl, Sigma-Aldrich, MO, USA) were combined with 50 µL of sample/standard. Each sample was analyzed in triplicate, while the standards were prepared in quadruplicate in transparent 96 well-plates (Corning-Costar Corporation, MA, USA) (Figure 10 and Figure 11). Supernatant fluorescence was

measured using a microplate reader (BioTek Synergy H1, BioTek Instruments, USA) at an excitation wavelength of 485/20 nm and emission wavelength of 528/20 nm. Upon acquiring the RFU values, it's essential to deduce the FMN concentrations (in µg/mL) for each sample. For that, a standard curve was prepared for FMN concentrations analysis with the following concentrations range.

2.2.1. Standard Stock Solution Preparation

Using 10 mg of Riboflavin 5'-monophosphate sodium salt hydrate (Sigma-Aldrich, MO, USA), the following stock solutions were prepared:

- Standard stock 1: 1 mg/mL;
- Standard stock 2: 10 µg/mL;
- Standard stock 3: 1 µg/mL.

Using the standard stock 3 solution, the following standard dilutions were prepared:

- S.A: 1 µg/mL (Derived from Stock 3);
- S.B: 0.5 µg/mL (500 µL Standard A + 500 µL ultrapure H_2O);
- S.C: 0.25 µg/mL (250 µL Standard A + 750 µL ultrapure H_2O);
- S.D: 0.125 µg/mL (125 µL Standard A + 875 µL ultrapure H_2O);
- S.E: 0.0625 µg/mL (62.5 µL Standard A + 937.5 µL ultrapure H_2O);
- S.F: 0.03125 µg/mL (31.25 µL Standard A + 968.75 µL ultrapure H_2O);
- S.G: 0 µg/mL (1000 µL ultrapure H_2O).

The calculations for the concentrations and volumes of the Stock solutions and Standards are detailed in the Section III of the Appendix.

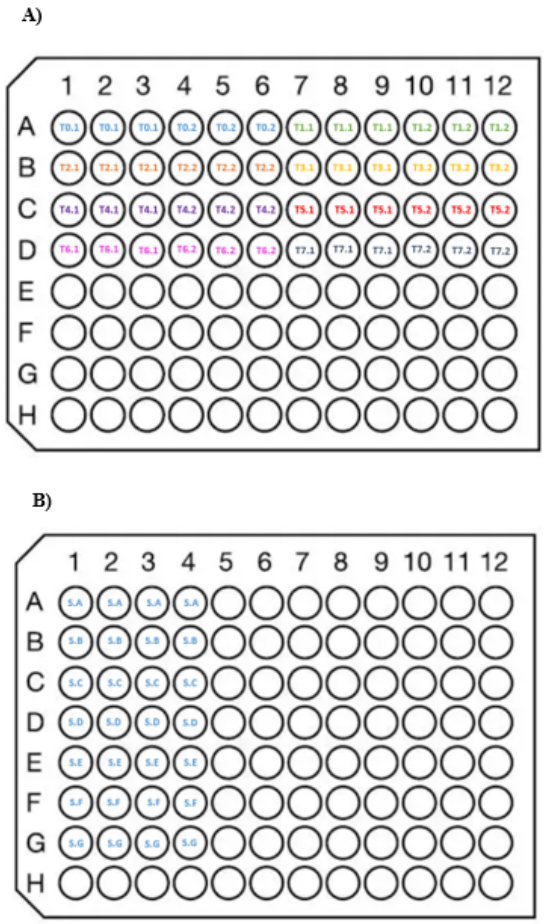

Figure 10 - 96-well plates diagram, illustrating the fluorescence reading of (A) samples and (B) standards using the microplate reader.

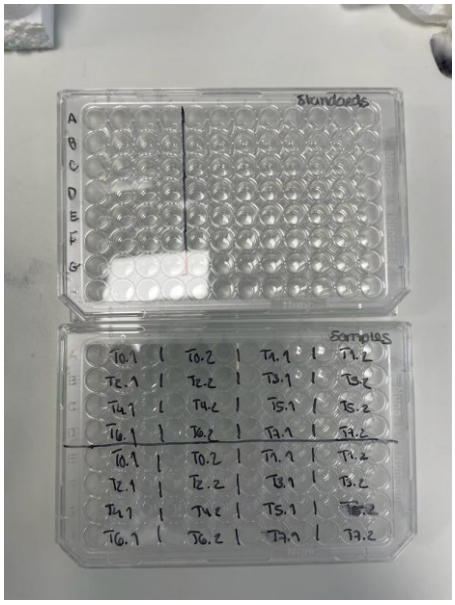

Figure 11 - Microplate reader used for samples measurement.

In the flowchart below (Figure 12) is presented a visual representation of the methodical flowchart that outlines the consecutive stages of the liver transplant procedure as executed in the research. Following the "Organ Retrieval", a "Organ Assessment" is conducted to determine whether the liver is suitable for transplantation. The subsequent step is the "HOPE" procedure, which was administered to 24 livers and is recommend to at least two hours. The "Liver Transplant" happens after the HOPE Procedure. "Perfusion Sample Collection" from 6 livers is a crucial branch from the HOPE stage that precedes the "FMN Quantification segment. This flowchart effectively summarises the sequential process of the study, from the organ retrieval to the application of FMN quantification in assessing liver viability.

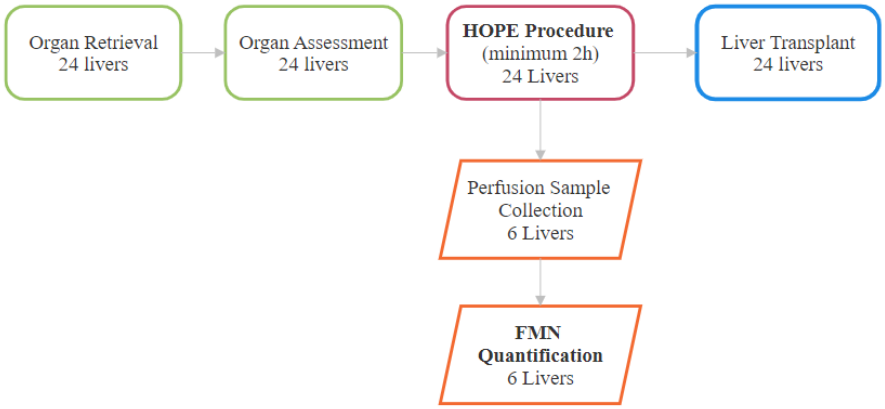

Figure 12 - Flowchart of the Liver Transplant Process with FMN Quantification in the Study.

4 RESULTS AND DISCUSSION

Since September 2022, CHUdSA has been successfully implementing the HOPE procedure for liver transplants. Up to the completion of the experimental work, a total number of 24 livers were treated with this innovative preservation technique, all of them analyzed in this thesis. Each of these treatments have been carried out without any complications or alarm, revealing safety and reliability in improving livers function prior transplantation and thus in implementing HOPE as a standard protocol at CHUdSA.

A critical issue of HOPE technique is the assessment of livers' viability during procedure, which has been explored in this study by measuring the FMN levels in the perfusate. FMN analysis is a relatively new contribution to the area of organ transplantation research, and although the existing literature on the subject is limited, it is expanding. As a result of recognizing the potential contribution of this analysis to the knowledge of organ preservation and transplant success, CHUdSA began collecting FMN samples in June 2023, which represents a noteworthy step into the field of transplant medicine in Portugal. However, since this study has only started in June 2023, the collection of FMN samples has been limited to the last 6 HOPE procedures carried out in CHUdSA.

Considering this, the results presented focus on the findings derived from the 6 liver transplants in which FMN analysis was conducted. Nonetheless, all 24 livers, without exception, were successfully transplanted, thus reaffirming the critical role of the HOPE procedure in improving outcomes related to organ preservation and transplantation at CHUdSA.

4.1 DONOR ANALYSIS

To ensure a comprehensive analysis of the results obtained in this work, the data concerning the 24 donors whose livers were submitted to HOPE have been carefully compiled as a standard protocol implemented by the hospital, which are presented in the experimental section of this thesis. The acquisition of these medical records was possible using the RPT – Registo Português de Transplantação, a specialized platform for the administration of transplant data management in Portugal.

The data contains a variety of significant information that is essential for understanding the context and potential impacts of HOPE procedure on transplant outcomes. Table 3 provide a

systematic presentation of the information regarding the 6 liver donor, whose perfusion fluid was collected during HOPE procedure and subjected to FMN analysis. This table represent a significant resource owing to their comprehensive and structured presentation of donor data, providing essential information for a holistic understanding and control of the entire transplant procedure.

Table 3 - Clinical Profile and Baseline Biochemical Parameters of Liver Donors for HOPE Procedure.

		Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
RPT Code		PT2023/000536	PT2023/000692	PT2023/000739	PT2023/000762	PT2023/000841	PT2023/000904
Age		72	66	61	76	45	70
Sex		Male	Male	Female	Male	Male	Female
Weight (kg)		75	64	62	85	80	55
BMI (Body Mass Index)		23.15	22.68	24.22	23.06	26.12	22.03
Date of Hospital Admission		04/07/2023	19/08/2023	12/09/2023	20/09/2023	14/10/2023	03/11/2023
Date of ICU Admission (Intensive Care Unit)		04/07/2023	19/08/2023	12/09/2023	20/09/2023	14/10/2023	03/11/2023
Duration of ICU Stay (Days)		2	3	2	2	4	5
Cause of Death		Hemorrahagic Stroke	TBI -Traumatic Brain Injury	Hemorrahagic Stroke	Hemorrahagic Stroke	Asphyxia (Anoxia)	Hemorrahagic Stroke
Baseline Value (Before Harvesting)	**AST (U/L)**	20	15	51	27	73	67
	ALT (U/L)	18	21	23	11	187	36
	Lactate (mmol/L)	1.10	1.83	1.43	*	1.0	0.7
	Total Bilirrubin (mg/dL)	0.86	1.08	0.56	0.37	0.70	0.37
	Creatinine (mg/dL)	1.04	0.92	0.67	1.18	1.4	1.13

*No value obtained.

As observed from Table 3, donors 1, 2, 4 and 6 comply with ECDs on account of their advanced age, surpassing 65 years. Donor 4 is particularly noteworthy, since it holds the distinction of being the oldest among the six donors discussed in this study. Conversely, donor 3, despite being younger that the traditional age limit for ECDs, the long period of cold ischemia to which the organ was subjected allowed to classify it as ECD. The inclusion of donor 5 was determined by the cause of death, which is asphyxiation, which led to an extended warm ischemia duration. This particular factor had a substantial impact on its classification, highlighting the importance of considering clinical events into the assessment of ECDs status.

Upon careful analysis of the baseline values prior to organ harvesting, all six donors demonstrated parameters that fell within the acceptable ranges, none of the displayed values that deviated from that acceptable range. This observation is of the utmost importance as it verifies that the biochemical status of all donor's livers remained within viable limits.

Nevertheless, it is critical to highlight a particular occurrence involving donor 2, who experienced cardiorespiratory arrest, which could significantly affect the quality of the donate organ.

4.2 HOPE PROCEDURE

As previously stated, the 24 transplants were carried out after HOPE procedure under the VitaSmart machine from *Bridge to Life* (Figure 13), and no complications were reported during the procedure. Following the criteria of the perfusion protocol that was established by the hospital and presented in the experimental section of this thesis, the perfusion time for each of the 6 transplants, was at least 2 hours. Table 4 illustrates the perfusion time for the 6 transplants, according to the data collected from the perfusion equipment.

Figure 13 - Liver under HOPE procedure.

Table 4 - HOPE Treatment Time of the six transplants.

HOPE Treatment	Duration (min)
Transplant 1	194
Transplant 2	134
Transplant 3	152
Transplant 4	148
Transplant 5	187
Transplant 6	130

As Table 4 shows, the HOPE procedure had an extended duration in transplants 1 and 5.

After 30 minutes of HOPE, the transplant recipient is transferred to the operating room where it is prepared for transplantation. During the surgery, called hepatectomy, certain complications may occur, including bleeding, lesions of the nearby organs due to the proximity to the liver or even complications derived from anaesthesia. By maintaining the liver in the perfusion equipment during this process, its preservation is ensured until the recipient is ready. So, if any complications occur during the hepatectomy, the liver can safely stay longer in HOPE. Therefore, this prolonged period of HOPE consists of a crucial adaptative measure, since it does not have significant consequences for the liver, allowing flexibility in the transplant process and above all, ensuring that the liver remains preserved for transplantation. Thus, it is of

extreme importance that hospitals are equipped of adaptable and robust systems like HOPE, that can preserve the viability of the organs even if anything unexpected happens during the complicated and sometimes unpredictable transplant procedure. As Table 4 shows, the HOPE treatment had an extended duration in transplants 1 and 5. This longer period of time was needed due to the unanticipated complications that occurred during the hepatectomy procedures of the recipients, specifically instances of uncontrolled bleeding. So, the livers remained connected to HOPE system while the surgical team meticulously addressed these complications. During the time required to stabilize the recipient's condition, this extended duration in HOPE was vital, as it ensured the preservation of the organ's viability. By following this approach, the livers were maintained in an ideal condition of preservation, making them immediately transplantable once the recipients were suitably prepared to undergo with the liver transplant procedure.

When the liver is maintained in the HOPE system for extended periods of time, as occurred with the livers from transplants 1 and 5, the strict maintenance of a stable and hypothermic temperature is an indispensable factor. HOPE operates under hypothermic conditions, which is a critical factor in mitigating the risk of mitochondrial injury, by decelerating metabolic processes, which lowers the risk of tissue damage and complications after the transplant and, therefore, improve the patient outcomes. However, in some situations, an unintentional increase in the temperature within the HOPE system, beyond the acceptable range, can potentially compromise the liver's functionality and integrity. In such conditions, immediate intervention is needed which can involve adjusting the equipment settings or adding ice in order to restore the temperature to the expected range. For this same reason and in conjunction with other factors, it is critical that the procedure is monitored by a trained health professional, including doctors, surgeons, nurses, or other health assistants.

4.3 RECIPIENT ANALYSIS

In addition to the clinical profile and baseline biochemical settings of the liver donors, it is imperative to rigorously assess the clinical parameters of each organ recipient. This assessment guarantees the transplant's successful completion and facilitates the early detection of any possible complications that may arise after the procedure. If complications do occur, this rigorous examination facilitates their prompt detection and control.

In order to acquire these crucial insights, SClinico, a comprehensive system designed for accessing and analyzing patient's clinical data and outcomes, was used. The data, which includes numerous clinical parameters, has been methodically gathered and is displayed in Table 5.

Table 5 - Clinical Profile and Post-Transplant Outcomes of Liver Recipients.

		Recipient 1	Recipient 2	Recipient 3	Recipient 4	Recipient 5	Recipient 6
Process Number		1947640	1904232	1928677	1948084	1931723	1849538
Age		51	60	58	56	61	54
Sex		Male	Male	Female	Male	Male	Female
Pathology		Alcoholic Cirrhosis	Hepatocellular Carcinoma	Alcoholic Cirrhosis	Alcoholic Cirrhosis	Hepatocellular Carcinoma	Primary Biliary Cirrhosis
Blood Type		A+	A+	O-	A+	B+	AB+
Date of Transplantation		06/07/2023	23/08/2023	14/09/2023	22/09/2023	18/10/2023	08/11/2023
Hospitalization Time (Days)		15	34	29	69	28	-
Baseline Value (Before Transplantation)	**AST (U/L)**	52	39	168	52	32	78
	ALT (U/L)	32	50	124	27	14	56
	Lactate (mmol/L)	1.7	1.2	4.2	*	*	*
	Total Bilirrubin (mg/dL)	1.6	1.03	3.40	11.08	1.22	27.47
	Creatinine (mg/dL)	0.56	0.86	0.52	1.22	0.71	0.90
Baseline Value (1 Day After Transplantation)	**AST (U/L)**	349	402	139	195	562	402
	ALT (U/L)	238	386	157	168	404	353
	Lactate (mmol/L)	2.2	3.2	1.6	1.3	4.5	1.0
	Total Bilirrubin (mg/dL)	6.43	3.28	5.85	8.76	6.44	6.84
	Creatinine (mg/dL)	1.41	0.65	0.83	1.39	1.14	1.36
Baseline Value (3 Day After Transplantation)	**AST (U/L)**	109	87	189	176	217	197
	ALT (U/L)	256	373	297	236	423	515
	Lactate (mmol/L)	0.9	1.6	0.4	0.9	1.1	1.0
	Total Bilirrubin (mg/dL)	4.38	1.15	11.12	3.60	4.90	4.63
	Creatinine (mg/dL)	0.97	0.81	0.56	1.67	1.30	0.77
Baseline Value (7 Day After Transplantation)	**AST (U/L)**	38	40	19	52	117	24
	ALT (U/L)	97	141	95	129	184	84
	Lactate (mmol/L)	0.6	*	*	1.0	1.2	*
	Total Bilirrubin (mg/dL)	3.97	0.95	9.58	3.25	5.70	3.86
	Creatinine (mg/dL)	0.45	0.70	0.48	1.03	0.76	0.43

*No value obtained.

Regarding **Recipient 1**, there was a notably increase in the levels of liver enzymes AST and ALT in the immediate post-transplant period, which peaked one day after transplantation. There was also an initial elevation of the lactate levels. However, by the third day these levels had returned to normal, suggesting that metabolic function and perfusion had improved. The peak in total bilirubin and creatinine levels at day one, possibly indicate temporary renal and hepatic stress, respectively. One week following the transplantation, a significant improvement was observed. Reduction in AST and ALT levels suggest a decrease in acute surgical stress and that the liver graft was operating efficiently. Approximately 1 month after the transplant, all biochemical levels had improved substantially and within the expected ranges, which indicate that the liver graft is operating normally and that there were no indications of hepatocellular injury o biliary flow obstruction. Overall, the data provides suggests that Recipient 1 has experienced a positive outcome subsequent to the transplant procedure.

Recipient 2, had a notable elevation in liver enzymes one day after liver transplantation, which could possibly indicate ischemia-reperfusion injury and surgical stress. The elevated lactate level is indicative of a temporary hypoxic state, possibly due to the surgery. However, by the 3rd day this level returns to normal which demonstrate an improvement in liver perfusion and metabolic function. A reduction in total bilirubin and creatinine levels in the days after transplantation is indicative of favorable liver graft and renal function. One week after transplant, liver enzymes started to return to normal levels, although the ALT remains elevated. In this case, it is crucial to continue monitoring the patient in order to identify possible complications. The prolonged hospitalization period of 34 days may be attributed to factors such as HCC (Hepatocellular Carcinoma) severity. Two months post-transplantation, all biochemical levels had improved and were well within the expected range. Also, the ALT levels returned to normal. This indicated that the liver graft is operating normally, and that the recipient has successfully recovered from the transplant.

In the case of **recipient 3**, the immediate post-transplant period was characterized by a notable increase in the liver enzymes AST and ALT, with particularly high lactate levels, which suggest that the patient was experiencing severe metabolic distress related to the metabolic cirrhosis. Total bilirubin levels increase significantly by the 3rd day which could be due to the liver adjusting to its new environment as well as the typical delay in functionality following the

procedure, also commonly referred to as “delayed graft function” or “initial poor function”. By the 7th day, there was a notable reduction in AST and ALT levels, suggesting that the transplanted liver is undergoing recuperation and resuming its functions within the recipients’ body. Nevertheless, the total bilirubin levels remain elevated which may be indicative of biliary complications or the continued dysfunction of the graft. Two months after transplantation, all liver functions tests have returned to normal (AST, ALT, and total bilirubin) and the creatinine levels were within the acceptable range, which indicates healthy graft function and absence of notable renal complications.

Regarding **Recipient 4**, there was a typical increase in AST and ALT levels one day after transplantation, which is indicative of surgical stress and liver’s reaction to IRI. However, on the 7th day after transplantation, these levels started returning to normal, which indicates recovery and adaptation of the liver graft. Lactate levels peaked on the first day but returned to normal within the initial few days following the surgery, suggesting and enhancement in the liver’s tissue perfusion and metabolic function. Prior to the transplant, this patient exhibited elevated total bilirubin levels which implied extensive cirrhosis-related liver dysfunction. Following the transplant, there was a significant reduction in total bilirubin levels, reflecting a potential return to normal levels and assuming enhanced hepatic functionally. Two months after transplant, the laboratory analysis showed normalized liver function levels and a significant improvement in total bilirubin.

In this case, it is important to highlight the fact that Recipient 4 has a prolonged hospitalization time of 69 days. This extended time was attributed to non-transplant-related complications.

Recipient 5, demonstrated at day 1 a significant increase in liver enzymes as a consequence of the liver response to the IRI and surgical stress. The lactate levels were also elevated, suggesting the presence of metabolic stress. However, this levels soon returned to normal, which is an encouraging sign of enhanced tissue oxygenation and improved liver function. The elevation in total bilirubin levels after transplantation may be assigned to the liver’s process of adapting to the new environment, as well as to initial compromised functionality or delayed graft function. By the 7th day, the AST and ALT levels has a slight decrease, indicating that liver stress was continuous but resolving. The total bilirubin levels still remained elevated and creatinine levels normalized, indicating renal recovery. One month after the transplant, the biochemical values

had substantially improved. The liver enzymes had normalized, which signifies that engraftment and function of the liver were accomplished successfully. Also, the total bilirubin levels came back to normal, and creatinine showed a slightly reduction comparing to pre-transplant values, indicating increased renal function. Overall, Recipient 5 had a positive outcome despite the initial fluctuations in AST, ALT and total bilirubin levels.

Recipient 6, has encountered significant difficulties during the post-transplantation phase. The day after the liver transplant, there was a considerable increase in the liver enzymes levels. Typically, surgical stress and IRI to the liver are responsible for this increase. Despite the normalization of these parameters by the 7th day, the overall course of events was disrupted by the development of bile duct stenosis, a serious complication that usually arises after liver transplantation. The total bilirubin levels prior to the surgery were significantly elevated, which is indicative of the severe liver dysfunction caused by the primary biliary cirrhosis. Following the transplant, even though these levels decrease, they failed to fully return to normal, which is concerning and likely attributable to the bile duct stenosis. This condition may result in bilirubin accumulation and impaired biliary flow. In relation to renal function, the initial elevation of creatinine suggested some renal stress or dysfunction. However, by the 7th day these levels decreased and returned to normal. Regardless of these advancements in the laboratory parameters, the patient was not discharged yet. This extended hospitalization is a result of the bile duct stenosis that has been compromising the patient's recuperation. This condition highlights the need for vigilant observation and frequently needs supplementary interventions.

Overall, this post-transplant results show a positive course of recovery and no signs of acute rejection or liver dysfunction.

4.4 FMN ANALYSIS

FMN analysis was performed in order to evaluate the viability of the livers intended for transplantation. The FMN analysis technique represents a significant innovation in the field of liver transplantation, primarily by offering an advanced method for assessing the viability of liver grafts (Eden et al., 2023; Huwyler et al., 2023). The quantification of FMN concentrations in the perfusion fluid throughout the HOPE procedure provides essential real-time insights regarding the mitochondrial state of the liver (Eden et al., 2023; Huwyler et al., 2023). Elevated concentrations of FMN in the perfusate are suggestive of reperfusion injury and mitochondrial damage, which may lead to graft dysfunction (Eden et al., 2023; Huwyler et al., 2023). The knowledge provided is of immense value to liver transplant surgeons, as it allows them to make well-informed judgements regarding the suitability of a liver for transplantation (Eden et al., 2023; Huwyler et al., 2023).

From the 24 livers subjected to HOPE procedure, only 6 were used for perfusate collection over time and analyzed by FMN (Figure 14). The calibration curves that enabled the determination of the FMN concentration values (Table 6) can be found detailed in Section IV of the Appendix.

Table 6 - FMN concentrations for the 6 post-HOPE transplanted livers.

Perfusion Time (min)	FMN Concentrations (µg/mL)											
	Liver 1		Liver 2		Liver 3		Liver 4		Liver 5		Liver 6	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
0	0,00548391	0,00723773	0,0029781630	0,002555062	0,007229904	0,010483806	0,005625240	0,008299680	0,010499145	0,010025532	0,005332455	0,006451904
5	0,00847824	0,00745161	0,0186329130	0,017692687	0,011152416	0,009858770	0,008968290	0,011776452	0,010757480	0,010886647	0,018851961	0,020832526
10	0,00993263	0,01108758	0,0253555230	0,027235973	0,008968290	0,014049727	0,007764792	0,007720218	0,012479710	0,011231093	0,021521418	0,022726979
15	0,01104481	0,01215699	0,0282702210	0,030009637	0,009770622	0,011330712	0,009681474	0,010394658	0,013426937	0,012436654	0,022038087	0,024190875
30	0,01048872	0,01450968	0,0317020430	0,034522718	0,009280308	0,010394658	0,009235734	0,009592326	0,014288052	0,014417219	0,022726979	0,025740882
60	0,01592129	0,01797455	0,0307061326	0,042755330	0,013024525	0,021850178	0,009815196	0,017704795	0,017861679	0,019411686	0,029486732	0,030132569
90	0,02011336	0,01891562	0,0513527490	0,047638860	0,014361745	0,012177618	0,011018694	0,011598156	0,015278334	0,019067240	0,035858984	0,043522908
120	0,02075500	0,02019891	0,0594856970	0,057699269	0,014896633	0,019978070	0,015698965	0,017660221	0,018938073	0,018378348	0,042446514	0,038657608

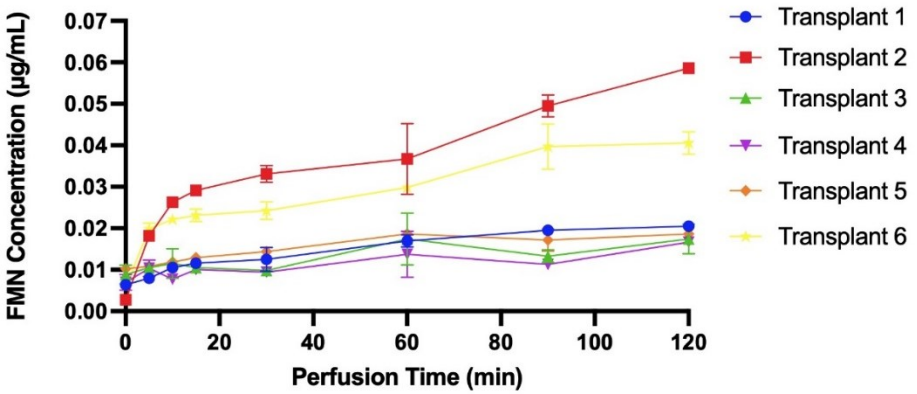

Figure 14 - FMN concentrations to analyse livers viability over 120 minutes of perfusion in HOPE.

The FMN quantification protocol is pioneer in Portugal and is still under validation at ESB-UCP, limiting the real-time analysis at CHUdSA where the transplants were conducted after HOPE. In light of the scarcity of literature in this domain, collaboration with the researcher Janina Eden at the Zurich Group was crucial in guaranteeing the precision of the results (Eden et al., 2023; Huwyler et al., 2023). According to previous results, FMN concentrations that in the first 60 minutes of HOPE surpass 0.03 μg/mL are suggestive of mitochondrial injury and indicate that the liver should not have been used for transplantation. Elevated concentrations of FMN indicate that the liver is releasing the FMN into the perfusate rather than retaining it, which is indicative of impaired liver function.

FMN values for Transplants 1, 3, 4, and 5 remained below the critical threshold indicating satisfactory metabolic activity in the liver, as shown in Figure 14. In agreement with these results, the recipients of these livers exhibited normalized post-transplant clinical profile and outcomes (Table 5), which are indicative of successful recoveries.

On the other hand, Transplant 2 demonstrated concentrations of FMN that were considerably higher than the established threshold. The donor in question had undergone cardiorespiratory arrest, which could have resulted in anoxia and consequent impairment of the liver's metabolic function. Despite this, the recipient recovered normally, which calls into question the predictive value of FMN in this instance. Nevertheless, due to the short time frame of this study, definitive conclusions regarding the implications of the elevated FMN levels cannot be drawn. It is imperative to conduct longer post-transplant follow-up, from three months to one year, to monitor late complications and possible need for retransplantation.

After 60 minutes perfusion, FMN concentrations approached the threshold for Transplant 6, which had no correlation with any known donor complications. However, post-transplant complications have been manifested into the recipient, specifically bile duct stenosis, which implies that this liver might have been suboptimal for use.

Transplants 2 and 6 serve as an illustration of the critical need for continuous and strict monitoring. It also underscores the drawback of exclusively depending on the outcomes reported by recipients after organ transplants to evaluate their viability. Thus, it is imperative to conduct a thorough assessment that includes donor clinical records as well as additional viability biomarkers (Eden et al., 2023).

This study shows that although recipient outcomes offer significant insights for post-operative care, they should not be the exclusive for organ viability evaluation. Despite normal clinical

outcomes, the recipient of Transplant 2 exhibited elevated levels of FMN, which highlights the importance of implementing real-time viability assessment methods (Eden et al., 2023).

In this study, it was presented the results regarding 6 liver transplants. However, it is important to note that a total of 24 transplants procedures were conducted throughout the study period up to the completion of this thesis, which underscores the fact that this sample size was comparatively restricted. Despite the encouraging results that were acquired, the present research work constitutes a preliminary study. It is imperative that forthcoming investigations should include a greater number of liver transplants to provide more comprehensive and conclusive data, thereby improving our comprehension of the effectiveness of the methodologies and processes herein utilized.

In summary, the FMN analysis is a critical biomarker in the field of liver transplantation, with the potential to serve as a universal indicator of the viability of the organ. The real-time analysis of FMN has the potential to improve the prognosis of livers viability and reduce post-transplant complications, thereby increasing the success rate of transplantation. This is only possible due to the implementation of HOPE perfusion, which maintains the stability of the pre-transplanted livers for longer periods sustaining the real-time analysis of FMN in the meantime. Thus, implementing the testing protocol near the operating room (i.e., acquiring the microplate reader to conduct the analyses) should be a priority for transplantation centers.

The long-time research in this field is demonstrated by the Zurich group (Eden et al., 2023), where investigation was first introduced in 2018 and has been shown interesting outcomes in FMN analysis (Eden et al., 2023). This research, encompassing a decade of liver transplants in Switzerland, highlights that donor-derived parameters alone may not be sufficient for estimating graft quality and predicting post-transplant outcomes (Eden et al., 2023). It also emphasizes the significance of implementing a meticulous approach to evaluate perfusate, which entails quantifying mitochondrial byproducts like FMN and NADH throughout HOPE (Eden et al., 2023). These biomarkers evaluate the quality of liver grafts and contribute to the determination of whether to implant or discard the organ (Eden et al., 2023). When considering this, FMN analysis assumes a particular importance because elevated concentrations in the perfusate indicate mitochondrial damage and may result in the rejection of a viable liver that was previously assessed macroscopically or according to donor data (Eden et al., 2023). The results from the Zurich Group study (Eden et al., 2023) underscore the importance of evaluating mitochondrial function to increase the viability of liver transplants, thereby potentially

decreasing the loss of viable livers, and improving the rate of successful transplants (Eden et al., 2023).

By integrating these discoveries into this study, the examination of FMN in this thesis corresponds to the groundbreaking endeavours delineated in the Zurich Group study (Eden et al., 2023). The significance of FMN quantification as a predictive biomarker for liver viability and its indispensable function in transplantation medicine are underscored. This analytical methodology has the potential to substantially reduce the number of discarded organs and enhance patient outcomes, constituting a significant advancement in the field. By taking advantage on these observations, this research has the potential to provide significant contributions to the continuous advancement of FMN as a universal biomarker and contribute to the improvements of transplant protocols to facilitate more informed clinical judgements.

The combination of FMN analysis and the HOPE procedure represents a significant and a turning point in the field of liver transplantation. By functioning as a biomarker for mitochondrial injury, FMN offers transplants teams instantaneous assessments of liver viability throughout HOPE, enabling them to make well-informed decisions regarding the transplantability of a given liver. This is particularly critical for livers obtained from ECDs, as they face an increased likelihood of being discarded. By enabling a more precise evaluation of which livers can recover function after transplant, this method has a potential to increase the number of viable organ donors and decrease their wastage. In addition to its technical advantages, this integration has a substantial effect on patient care. By utilising FMN analysis in HOPE, transplant waiting times can be reduced, addressing the organ shortage crisis problem, and post-transplant outcomes can be enhanced and the quality of live from the recipients of the transplant can be improved.

5. CONCLUSIONS AND FUTURE WORK

In the present thesis the effectiveness of the HOPE procedure for maintaining and improving the viability of liver grafts, particularly from ECDs which otherwise would be discarded from transplantation programs, was investigated and the validation of FMN as predictive biomarker of liver viability was achieved.

In the context of liver transplantation, HOPE procedure provides significant advantages in organ preservation, specifically when ECD are involved. In the past, livers obtained from ECDs were frequently considered unsuitable and discarded. In this way, the implementation of HOPE has allowed the effective utilization of ECDs, thereby expanding the pool of potential donors, and decreasing the waiting period for transplants.

In our study, the 24 recipients exhibited extremely favorable outcomes following these transplants, with no retransplantations required during the study period. A notable outcome was that, in comparison to previous transplant procedures that solely utilized SCS before the introduction of HOPE at CHUdSA, the incorporation of HOPE lead to reduced hospitalization periods and accelerated stabilization of transaminase levels. This highlights the effectiveness of HOPE, which is especially remarkable considering that it involved organs from donors who met broader criteria, ECDs. The results of this study demonstrate that HOPE is capable of successfully using organs that were previously considered inappropriate for transplantation, resulting in positive and successful transplantation. Although it is recognized that complications may arise after transplants even when HOPE is implemented, and patients are not completely protected from the common transplantation risks, the overall trend suggests that outcomes are considerably better when HOPE is utilized as opposed to situations in which is not utilized.

An innovative element of this work is FMN biomarker quantification, as an additional validation tool, which represents a substantial advancement in transplant viability research in Portugal. Our study revealed that the recipient's clinical outcomes and donor organ viability, indicated by FMN levels, were well correlated and encouraging. However, the short timeframe of the present investigation and contradictory results when related to the clinical outcomes of recipients, did not allow us to fully authenticate FMN as a predictive biomarker. Thus, it is crucial to keep a long-term surveillance of transplant recipients in order to validate with increased accuracy FMN as a predictor of successful transplantations. Moreover, it is important

to emphasize that the FMN quantification analysis conducted in this study was limited to only 6 of the 24 livers that underwent the HOPE procedure. Although it would have been optimal to perform the FMN analysis on all 24 livers, logistical limitations prevented this occurrence. In the future it is critical to further develop and broaden this study to encompass more livers that have been treated with HOPE. By doing this, the sample size can substantially increase, to guarantee the robustness of the method. By increasing the sample pool of this analysis, a more extensive set of data will be obtained, thereby allowing more definitive insights regarding the effectiveness of FMN as a predictive biomarker of liver viability. By establishing this quantitative predictive method for assessing liver viability in Portugal, the potential of transplant medicine would certainly advance. The simple acquisition of appropriate equipment to read FMN levels in the perfusate would revolutionize the transplant centers allowing for a real-time viability assessment and the provision for faster and more reliable information to clinicians during the transplant process.

Future research should also be dedicated to expand the functionality of HOPE to encompass additional organs, with particular emphasis on the kidney. There is reason to be optimistic regarding the potential of HOPE to enhance kidney transplant outcomes in the same way liver transplants have achieved encouraging results. The subsequent phases will involve adapting the protocols and techniques proven effective in liver transplantation to kidney transplants at CHUdSA, potentially setting a new standard for organ preservation for kidney. This expansion has the potential to profoundly affect both the quantity of viable organs and the post-operative well-being of recipient patients, thereby establishing a new standard in the field of transplant medicine on a global scale.

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APPENDIX

Section I

Table 7 - Composition of preservation solutions. Retrieved from (Guibert et al., 2011b).

Component	Euro-Collins	UW (Viaspan®)	Celsior®	Custodiol®	IGL-1®
NaCl	10.0	–	–	15.0	–
KCl	108.0	–	15.0	9.0	–
Potassium hydrogen 2-ketoglutarate	–	–	–	1.0	–
Lactobionate	–	100.0	80.0	–	100.0
PEG 35,000	–	–	–	–	0.03
$MgCl_2$ 6 H_2O	–	–	13.0	4.0	–
$NaCO_3H$	10.0	–	–	–	–
NaOH	–	25.0	100	–	*
KOH	–	100.0	–	–	–
$MgSO_4$	–	5.0	–	5.0	5.0
Glutamic acid	–	–	20.0	–	–
Glutathione	–	3.0	3.0	–	3.0
Glucose	180.0	–	–	–	–
Histidine HCl H_2O	–	–	30.0	18.0	–
Histidine	–	–	–	180.0	–
Adenosine	–	5.0	–	–	5.0
Allopurinol	–	1.0	–	–	1.0
Tryptophan	–	–	–	2.0	–
Mannitol	–	–	60.0	30.0	–
Raffinose	–	30.0	–	–	30.0
$CaCl_2$ 2 H_2O	–	–	0.25	0,015	0.03
Pentafraction (HES), g/l	–	50.0	–	–	–
Phosphate	60.0	25.0	–	–	25.0
Insulin, U/l	–	40.0	–	–	–
Dexamethasone, mg/l	–	16.0	–	–	–
Penicilin G, UI/l	–	200,000	–	–	–
Na^+	10.0	25.0	100.0	15.0	125.0
K^+	115.0	125.0	15.0	10.0	30.0
pH	7.30 (0 °C)	7.40 (25 °C)	7.30 (20 °C)	7.02–7.20 (25 °C)	7.40 (25 °C)
Osmolality, mosmol/kg H_2O	340	320	242–368	310	320

[a]Constituents of five commonly used and commercially available preservation solutions. Values are shown as mmol/l unless otherwise indicated.
*Necessary amount to adjust pH value.

Section II

santo antónio

CENTRO HOSPITALAR UNIVERSITÁRIO DE SANTO ANTÓNIO

Protocolo – Perfusão de Órgãos (HOPE)

1. Objetivo/Enquadramento

O transplante de órgãos consiste no tratamento utilizado para doentes que sofrem de falência de órgãos em estágio terminal, sendo considerado um dos principais avanços na medicina moderna, dado que proporciona os melhores resultados em termos de mortalidade, morbidade e qualidade de vida do paciente, sendo, normalmente, a única hipótese de sobrevivência do mesmo. Contudo, o transplante de órgãos enfrenta inúmeros desafios, sendo o principal o desequilíbrio entre a diminuição do número de órgãos disponíveis e o número de pessoas em lista de espera que necessitam de transplante. Como consequência, do elevado número de candidatos nas listas de espera e do tempo de espera, há um agravamento do estado geral dos pacientes que estão à espera do órgão, bem como um aumento do número de complicações pós-transplante. Estudos estatísticos demonstram que em os órgãos mais transplantados a nível mundial, bem como a nível nacional, consistem no rim e no fígado.

Tendo todos estes fatores em consideração, surge a necessidade de mitigar estes obstáculos associados ao transplante de órgãos e melhorar a qualidade de vida do órgão pós-transplante. Cada vez mais os dadores de órgãos apresentam critérios expandidos (idade, comorbilidades, esteatose, doença vascular e dadores em paragem circulatória) que aumentam o risco de síndrome de reperfusão severa no recetor, bem como complicações adicionais no pós-transplante, o que se traduz num aumento dos custos de cirurgia devido ao aumento da demora média na unidade de cuidados intensivos. Deste modo, é crucial que sejam tidos em consideração os avanços realizados no âmbito da engenharia biomédica que permitem colmatar estes obstáculos e assim melhorar a qualidade de vida do órgão e consequentemente, do paciente. Um dos novos avanços na área é relativo ao sistema de perfusão de órgãos, nomeadamente a tecnologia HOPE – Hypothermic Oxygenated Machine Perfusion, recorrendo ao equipamento VitaSmart. Este equipamento consiste num sistema de perfusão onde o fígado é colocado após a cirurgia de colheita e imediatamente antes do transplante, sendo realizada a sua perfusão através da canulação da veia porta. Através da utilização deste sistema de perfusão, é possível promover a reparação do enxerto, o seu reacondicionamento e a sua imunomodulação. Em acréscimo, esta tecnologia de reacondiciomento de órgãos facilita a manutenção do tônus microvascular do órgão, potencia a oxigenação para o metabolismo dos tecidos e remove todos os resíduos metabólicos.

2. Âmbito

Condicionamento por perfusão oxigenada hipotérmica dos enxertos hepáticos

3. Definições/Evidências

HOPE ou Hypothermic Oxygenated Machine Perfusion consiste numa técnica utilizada frequentemente para preservar e melhorar a função dos órgãos pós-transplante, sendo utilizada principalmente ao nível do fígado e do rim. Inicialmente, o órgão é colocado numa bacia estéril com a temperatura a ser controlada ao longo de todo o processo, sendo o órgão perfundido com uma solução específica que contém o oxigénio, nutrientes e outros componentes necessários à preservação do órgão.

O procedimento HOPE é geralmente realizado num intervalo de temperatura entre 4 a 12ºC, o que se traduz no atraso no metabolismo do órgão e, consequentemente, reduzindo o risco de danos nos tecidos. De facto, a solução oxigenada permite que sejam fornecidos o oxigénio e os nutrientes essenciais à sobrevivência do órgão, melhorando a sua função e reduzindo o risco de falência do mesmo pós-transplante. Adicionalmente, HOPE também ajuda a reduzir os danos que podem ocorrer durante o processo de remoção de órgão do dador até ao seu implante no recetor.

A HOPE tem sido uma técnica cada vez mais popular e utilizada no âmbito do transplante hepático e renal, dado que demonstrou melhorar positivamente os resultados do transplante e aumentar o número de órgãos que podem ser utilizados para transplante, principalmente ao nível da utilização de dadores de critérios expandidos.

4. Responsabilidades

Diretor Programa de Transplantação Hepática

Gabinete de Coordenação de Colheita e Transplantação

Bloco operatório

5. Metodologia

A. Critérios de Aceitação de Dador

Critérios expandidos:

- Idade ≥ 65 anos
- IMC ≥ 30 Kg/m2
- Bilirrubina total máxima ≥ 2.0 mg/dL
- AST máxima ≥ 500 U/L
- ALT máxima ≥ 500 U/L
- Dador de coração parado
- Tempo de isquemia fria > 10 horas
- Uso ≥ 2 vasopressores em qualquer momento
- Ventilação mecânica > 5 dias
- Esteatose > 30%

Procedimento habitual na cirurgia de colheita e no acondicionamento e transporte do órgão

B. Utilização da VitaSmart

Critérios mínimos para aceitação da utilização da máquina

Tempo de perfusão mínimo de 2h

Variação de temperatura mínima e máxima (4ºC-12ºC)

Volume de líquido de perfusão 2-3L (dependendo da volumetria do enxerto)

Fluxo máximo 250 mL/min

Pressão máxima 3 mmHg

Procedimento de lavagem e fase de perfusão – Guia VitaSmart (Anexo)

Colher 3 amostras durante o tempo de perfusão (T0- 0 min, T1- 15 min, T2 – 30 min, T3- 60 min, T4 – fim) – preencher tabela

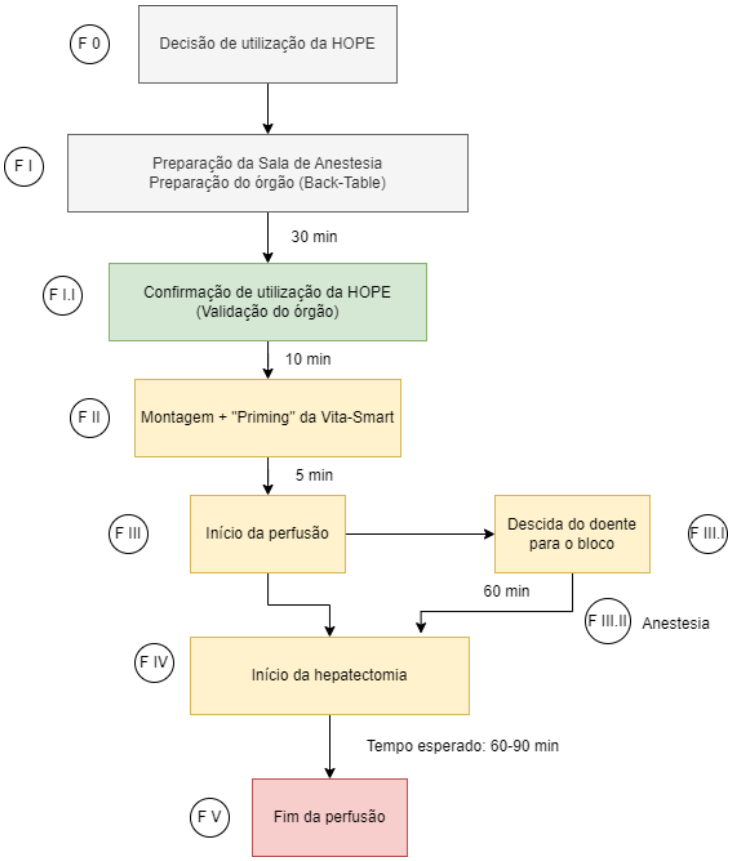

Figure 1 – Fluxograma da utilização da HOPE.

D. Colheita de dados para análise

Existência de síndrome de reperfusão após implantação do enxerto

Colher análises do recetor no 1º, 3º e 7º dia pós transplante de TGO, TGP, bilirrubina total, lactato e creatinina

Critérios de disfunção precoce e tardia do enxerto

Complicações vasculares (trombose da artéria hepática)

Tempo de internamento e tempo de cuidados intensivos

Complicações biliares tardias

Retransplante

Sobrevida do enxerto

Em fase de aquisição: doseamento de flavina de mononucleótido (FMN)

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santo antónio

CENTRO HOSPITALAR UNIVERSITÁRIO DE SANTO ANTÓNIO

Protocolo – Estudo da Viabilidade dos fígados (Análise do FMN)

1. Objetivo/Enquadramento

Atualmente, um dos principais obstáculos da atividade de transplantação é a discrepância entre a quantidade de órgãos disponíveis para transplante e o número de pessoas em lista de espera. No sentido de diminuir esta discrepância e aumentar o número de órgãos disponível para transplante surge o conceito de dadores de critérios expandidos (idade, comorbilidades, esteatose, doença vascular e dadores em paragem circulatória) que aumentam o risco de síndrome de reperfusão severa no recetor, bem como complicações adicionais no pós-transplante, o que se traduz num aumento dos custos de cirurgia devido ao aumento da demora média na unidade de cuidados intensivos. Assim, tendo todos estes fatores em consideração, no ramo da engenharia biomédica têm sido conduzidas investigações relativamente a diferentes técnicas de perfusão, nomeadamente a tecnologia HOPE – Hypothermic Oxygenated Machine Perfusion, recorrendo ao equipamento VitaSmart. No entanto, apesar desta tecnologia ter outcomes extremamente positivos no que toca a durabilidade do órgão e o número de compliações pós-transplante, é importante que seja avaliada a viabilidade do órgão pré-transplante de modo a reduzir, não só, as complicações pós-transplante bem como, o número de órgãos elegíveis. Contudo, não existe nenhum biomarcador que permita avaliar de forma rigorosa e precisa a viabilidade do órgão e assim, auxiliar o processo de decisão antes da implantação do órgão, pelo que é necessário que sejam investigadas e estudadas novas metodologias de avaliação confiáveis e rigorosas. Mais recentemente, tem sido utilizado o mononucleótido de flavina (FMN) como biomarcador preditivo da viabilidade do fígado durante a utilização da tecnologia HOPE após um período de isquemia.

2. Âmbito

Condicionamento por perfusão oxigenada hipotérmica dos enxertos hepáticos

Estudo da viabilidade dos enxertos hepáticos após perfusão hipotérmica oxigenada

3. Definições/Evidências

O fígado consiste num órgão metabolicamente muito ativo, sendo que uma componente essencial ao seu metabolismo consiste na criação e utilização de energia na forma de trifosfato de adenosina (ATP). Vários processos metabólicos, incluindo a oxidação de coenzimas reduzidas como o mononucleótico de flavina (FMN) e a dinucleótido de nicotinamida e adenina (NADH) são utilizados para produzir essa mesma energia. Neste momento, no âmbito do estudo da preservação e viabilidade de órgãos pré e pós transplante, vários investigadores têm analisado a libertação de FMN do fígado para o líquido de perfusão durante a utilização da HOPE como um marcador para avaliar ischemia-reperfusion injury (IRI) – síndrome de reperfusão.

O FMN consiste numa pequena molécula que está associada ao complexo mitocondrial I (C-I) que contribui para a produção de energia, sendo um componente essencial da cadeia de transporte de eletrões, responsável por aceitar os eletrões do NADH. A molécula de FMN reduzida enfraquece a sua conexão com o complexo mitocondrial I durante o tempo de isquemia (perda de suprimento de oxigénio e sangue que afeta o fígado), sendo que esta falha na conexão prejudica a atividade C-I e interfere na síntese de ATP e no fluxo de eletrões.

Durante o procedimento HOPE, o fígado é perfundido com uma solução oxigenada enquanto é colocado num ambiente regulado de baixas temperaturas (4 a 12ºC). Este processo tem como objetivo proteger e melhorar a viabilidade do órgão antes do transplante. Contudo, durante a HOPE, a reposição de oxigénio e nutrientes pode resultar num dano IRI, uma vez que a reperfusão do fígado conduz a um pico de atividade metabólica e, consequentemente, a libertação de FMN e NADH das mitocôndrias danificadas.

A quantidade do dano causado por IRI e a disfunção mitocondrial do fígado são indicadas pela libertação de FMN para o líquido de perfusão. Assim, neste momento, ainda que bastante preliminares, têm sido conduzidos estudos que visam avaliar a gravidade do IRI e prever os níveis de FMN do líquido de perfusão após um determinado período de perfusão hipotérmica. De facto, vários estudos demonstraram uma relação entre os níveis de FMN no líquido de perfusão após 30 minutos de HOPE e o tempo de internamento pós-transplante, número total de problemas e a probabilidade de falha do enxerto em 90 dias.

Assim é importante considerar que:

- Níveis altos de FMN no líquido de perfusão mostram que o FMN está a ser libertado do fígado, indicando lesão ou possível dano hepático, uma vez que os níveis de FMN no complexo C-I no fígado, o que indica que a atividade metabólica do fígado se encontra comprometida.
- Níveis baixo de FMN no líquido de perfusão são vistos como um indicador positivo de viabilidade do órgão, uma vez que o FMN não está a ser libertado do fígado, estando a ser utilizada pelo mesmo, sendo os níveis de FMN altos no fígado. Isto indica que a atividade metabólica do fígado está a funcionar corretamente.

4. Responsabilidades

Diretor Programa de Transplantação Hepática

Gabinete de Coordenação de Colheita e Transplantação

Bloco operatório

Equipe cirúrgica de apoio à transplantação

5. Metodologia

O líquido de perfusão a ser utilizado deve ser o Belzer MPS.

1) Colher amostras do líquido de perfusão para os seguintes tempos:
    - T0 – 0 min (início de HOPE – valor de controlo)
    - T1 – 5 min após o início de HOPE
    - T2 – 10 min após o início de HOPE
    - T3 – 15 min após o início de HOPE
    - T4 – 30 min após o início de HOPE
    - T5 – 60 min após o início de HOPE

Devem ser colhidas 2 amostras para cada um dos tempos definidos acima, dando um total de **16 amostras**, sendo cada uma delas colhidas em tubos secos de 1 mL (Eppendorfs).

Cada tubo deve estar **identificado com o tempo de colheita**.

Cada tubo deve ser armazenado em **gelo e protegido da luz**, pelo que os tubos Eppendorf devem estar envolvidos por papel de alumínio.

2) Após a colheita, as amostras devem ser colocadas em gelo picado, protegidas da luz e acondicionadas numa caixa isotérmica.

3) Transportadas depois para frigorífico a -20ºC na Escola Superior de Biotecnologia da Universidade Católica Portuguesa.

4) As amostras irão ser trabalhadas e analisadas utilizando o equipamento de leitura de microplacas Escola Superior de Biotecnologia da Universidade Católica Portuguesa, previamente calibrado segundo o protocolo de calibração fornecido pela Bridge to Life.

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Section III

Stock Solution Preparation

Using 10 mg of riboflavin, the following stock solutions were prepared:

- **Stock 1: 1mg/mL**

  10 mg Riboflavin + 10 mL H_2O ultrapure = $\frac{10\ mg}{10\ mL} = 1\frac{mg}{mL}$

- **Stock 2: 10 µg/mL**

  $ci\ x\ vi = cf\ x\ vf \Leftrightarrow 1\frac{mg}{mL}\ (Stock\ 1)\ x\ vi = 10\ \frac{\mu g}{mL} x\ 10\ mL \Leftrightarrow vi = 100\ \mu L$

  100 µL Stock 1 + 9,90 µL H_2O ultrapure → 10 µg/mL

- **Stock 3: 1 µg/mL**

  $ci\ x\ vi = cf\ x\ vf \Leftrightarrow 10\frac{\mu g}{mL}\ (Stock\ 2)\ x\ vi = 1\ \frac{\mu g}{mL} x\ 10\ mL \Leftrightarrow vi = 1\ mL$

  100 mL Stock 2 + 9 mL H_2O ultrapure → 1 µg/mL

Standards Formulation

- **A: 1 µg/mL (Derived from Stock 3)**

- **B: 0,5 µg/mL (500 µL A + 500 µL ultrapure H_2O)**

  $ci\ x\ vi = cf\ x\ vf \Leftrightarrow 1\ \frac{\mu g}{mL}\ x\ vi = 0{,}5\ \frac{\mu g}{mL}\ x\ 1\ mL \Leftrightarrow vi = 0{,}5\ mL$

  500 µL Standard A + 500 µL H_2O ultrapure → 0,5 µg/mL

- **C: 0,25 µg/mL (250 µL A + 750 µL ultrapure H_2O)**

  $ci\ x\ vi = cf\ x\ vf \Leftrightarrow 1\ \frac{\mu g}{mL}\ x\ vi = 0{,}25\ \frac{\mu g}{mL}\ x\ 1\ mL \Leftrightarrow vi = 0{,}25\ mL$

  250 µL Standard A + 750 µL H_20 ultrapure → 0,25 µg/mL

- **D: 0,125 µg/mL (125 µL A + 875 µL ultrapure H_2O)**

  $ci\ x\ vi = cf\ x\ vf \ \Leftrightarrow 1\ \frac{\mu g}{mL}\ x\ vi = 0{,}125\ \frac{\mu g}{mL}\ x\ 1\ mL \ \Leftrightarrow vi = 0{,}125\ mL$

  125 µL Standard A + 875 µL H_2O ultrapure → 0,125 µg/mL

- **E: 0,0625 µg/mL (62.5 µL A + 937.5 µL ultrapure H_2O)**

  $ci\ x\ vi = cf\ x\ vf \ \Leftrightarrow 1\ \frac{\mu g}{mL}\ x\ vi = 0{,}0625\ \frac{\mu g}{mL}\ x\ 1\ mL \ \Leftrightarrow vi = 0{,}5\ mL$

  62,5 µL Standard A + 937,5 µL H_2O ultrapure → 0,0625 µg/mL

- **F: 0,03125 µg/mL (31.25 µL A + 968.75 µL ultrapure H_2O)**

  $ci\ x\ vi = cf\ x\ vf \ \Leftrightarrow 1\ \frac{\mu g}{mL}\ x\ vi = 0{,}03125\ \frac{\mu g}{mL}\ x\ 1\ mL \ \Leftrightarrow vi = 0{,}03125\ mL$

  31,25 µL Standard A + 968,75 µL H_2O ultrapure → 0,03125 µg/mL

- **G: 0 µg/mL (1000 µL ultrapure H_2O)**

Section IV

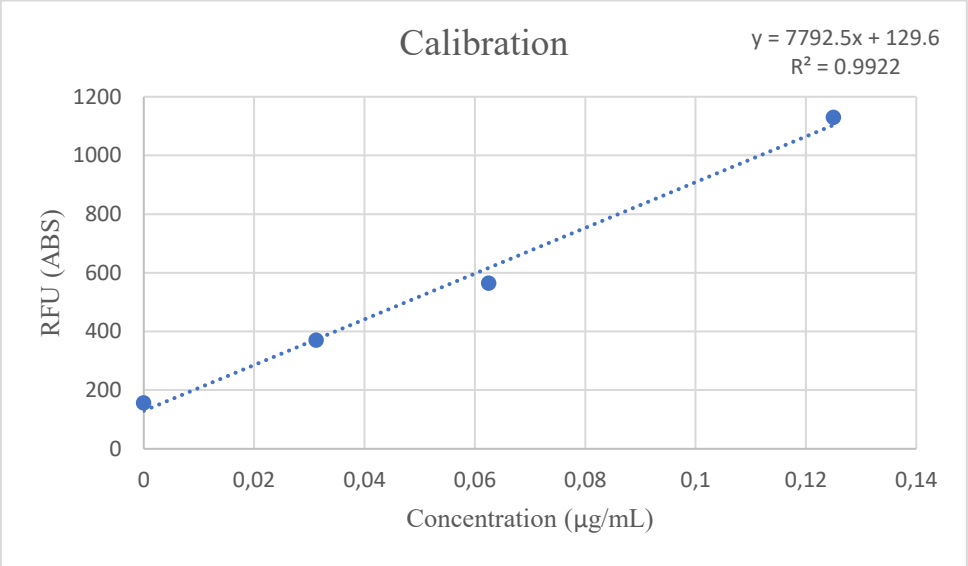

Figure 15 - Calibration curve regarding the standards values for Transplant 1.

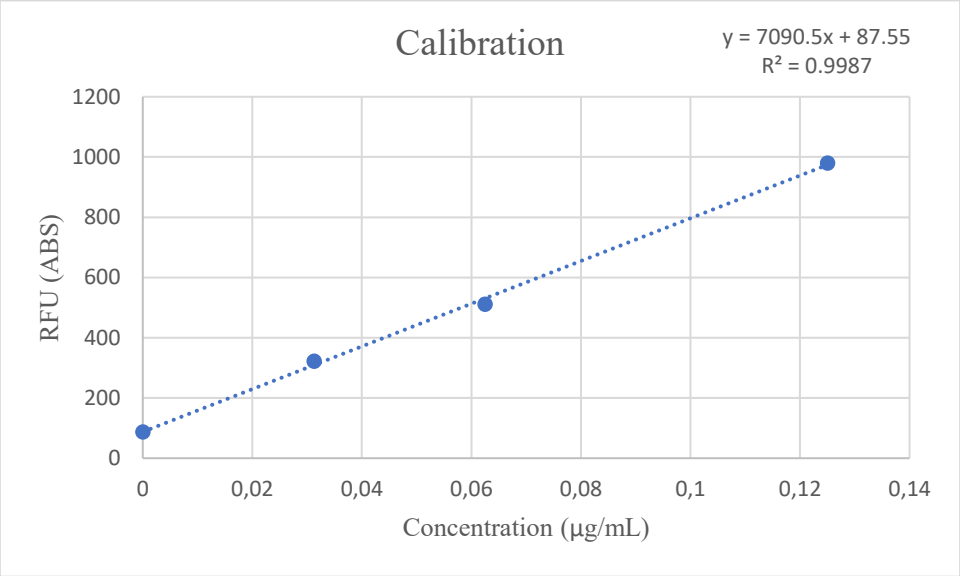

Figure 16 - Calibration curve regarding the standards values for Transplant 2.

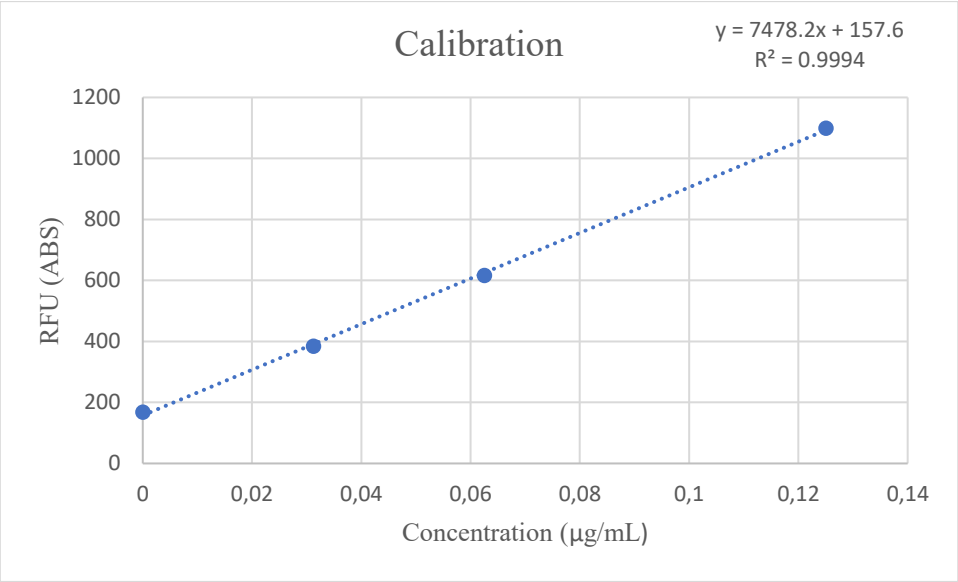

Figure 17 - Calibration curve regarding the standards values for Transplant 3 e 4.

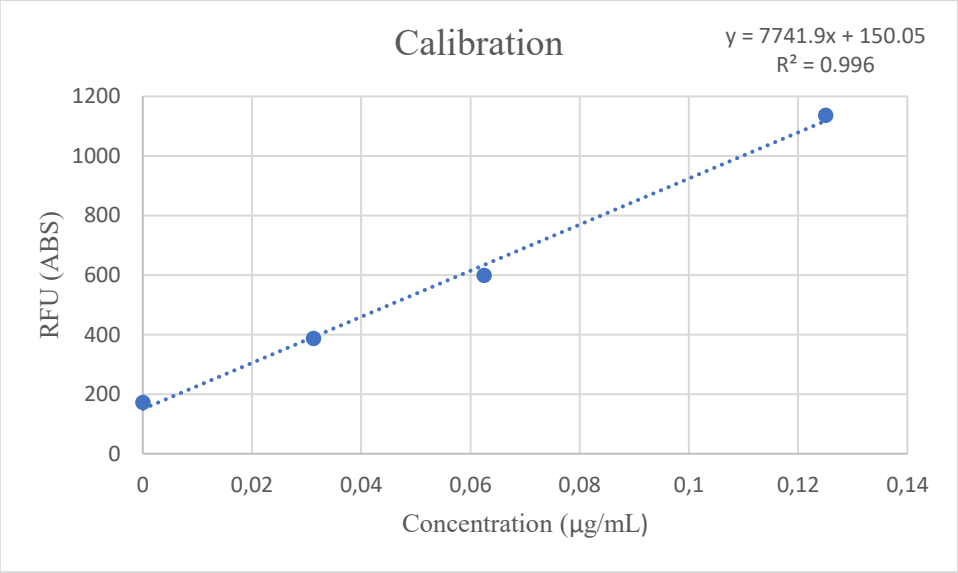

Figure 18 - Calibration curve regarding the standards values for Transplant 5 e 6.