

NRF2 in liver injury and repair: its relevance to liver transplantation



This thesis is submitted to Trinity College Dublin, the University of
Dublin, for the degree of Doctor of Philosophy (Ph.D) by

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2023

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Ethical approval was acquired for all experiments involving human and animal samples. For all experiments involving human tissue, written and informed consent was obtained. All animal experiments were carried out under correct permits and humane conditions.

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Acknowledgments

This thesis is not just my work. I am deeply indebted to many talented individuals who have supported my research journey and while I could never repay everyone for their time and guidance, I hope to at least pay this forward during my career.

First and foremost, my heartfelt thanks go to my supervisor Professor Cliona O'Farrelly. Like most PhD students, I may have underestimated the difficulty of this experience from the start and making the transition from a surgical trainee to a scientific researcher was, at times, intimidating. For this reason, I could not have asked for a better academic supervisor. Prof's expert advice and wisdom have taught me to think outside the box, always aiming to look further and dig deeper. Because of her, my journey became more than just bench work and data analysis. She has given me the confidence to aim beyond this and to look ahead at how this work can help real life patients. I was truly blessed to have a supervisor who genuinely wanted to bring out the best in me. Becoming part of her lab group has also given me the pleasure of meeting many other wonderful researchers who have been equally kind and patient with me. A special thank you to Jamie, Grainne and Mihai for, not only reading but, actually responding to my long whiny emails and reassuring me that "it'll all come together"!

Professor William Chapman has been a huge inspiration to me in both an academic and surgical capacity. I had the pleasure of first meeting him during my final year student elective in 2013 and, as fate would have it, I would be returning just 6 years later to join his group as a research fellow. Without his support, this work would not be possible. Through his mentorship I have learned not to shy away from challenging accepted thinking and to always ask "*how is this going to change practice?*". The unit he has created in Barnes and the teaching environment he has built has, not only inspired me but, laid the blueprint for generations of trainees, residents and fellows to follow. I can only aspire to his insight and vision. I would also like to thank the rest of Professor Chapman's lab for their guidance during my time in WUSM. I am especially grateful to Dr Min Xu, an exceptionally intelligent academic, who was always available for advice. Min was a constant source of support, even spending his weekends and days off with me in

the lab explaining scientific principles, teaching me relevant lab techniques and listening to me moan about my failed experiments! I wish him all the best in his career. I also wish to acknowledge that some of these experiments were funded, in part, by the Barnes-Jewish Hospital Foundation Project Award, Transplant Research Support, Mid-America Transplant's Clinical Innovation Fund and by the National Institutes of Health grant 5R44DK092078. I am forever grateful to all the donors.

There are a number of clinical mentors who have hugely influenced my career and I would not be here today without them. Thank you to Mr Ken Mealy, not only for his generosity during my time in Wexford (and for introducing me to Prof O'Farrelly), but for his career and research guidance. Through Mr Mealy, I developed an interest in public health outcomes, service access and large scale real-world issues, contributing significantly to the foundations of this current work. Professor Majella Doyle is one of the best role models I have ever met. Her career is one I can only aspire to and she has encouraged me to think bigger and aim higher in all facets of life. Although the Covid-related travel restrictions to Ireland were stressful at times, I never felt alone and Ladue became my home away from home. I couldn't have survived my time in St Louis without her.

It is difficult to overstate my gratitude to Mr Gerry McEntee. Since Res year, knowing him has been one of the biggest honours of my career. His insight, encouragement and advice were and continue to be invaluable. I have not been the best at expressing my appreciation to him over the years, but I owe him more than I could ever repay or ever write in a PhD thesis. He has inspired me greatly since med school to become the best that I can be and is, without a doubt, one of my biggest influences. Thank you for being the best example of what an operator, teacher and mentor look like.

I am grateful to all my friends for listening to me go on and on and on about liver cells and machine perfusion for the past 3 years. Thank you especially to Mary, Carol and Grainne for always thinking of me while I was living the expat life out Missouri and couldn't get back home. I was never short on Barrys tea, Tayto and Cadburys chocolate because of them!

My family were and continue to be a source of support during my studies. My mother Layla has always been my rock and gave me the confidence to venture out into the world, always telling me to reach for the stars. Thank you to my siblings – my big brother Ahmed, my younger sister Doua, my younger brother Omer and my baby sister Razan. I am grateful for your encouragement during all my time studying and working and thank you for holding things down when I was away.

Finally, I'd like to thank all the patients I have met on this journey and those I have yet to encounter. Thank you especially to all the donors and their families who have given the most important gift of all, the gift of life. I dedicate this thesis to you.

"If I have seen further it is by standing on the shoulders of Giants" – Sir Isaac Newton

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List of Abbreviations

Abbreviation	Definition
ALD	Alcoholic liver disease
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AU	Arbitrary Unit
BBN	Bladder-specific carcinogen N-nitrosobutyl(4-hydroxybutyl)amine
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CDDO	1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole
CIT	Cold ischaemia time
CK	Creatinine kinase
CLF	Chronic liver failure
CVA	Cerebrovascular accident
CXCL	Chemokine (C-X-C motif) ligand
DADLE	D-Ala ² , D-Leu ⁵ -Enkephalin
DAMP	Damage-associated molecular patterns
DAPI	Diamidino-2-phenylindole
DBD	Donation after brain death
DCD	Donation after cardiac death
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRI	Donor risk index
EASL	European Association for the Study of the Liver
ECD	Extended criteria donor

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EU	European Union
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCL	Glutamate cysteine ligase
GCLC	Glutamate cysteine ligase catalytic
GDPR	General Data Protection Regulation
GSH	Glutathione
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF	Hypoxia-inducible factors
<i>HLA</i>	Human leukocyte antigens
HMP	Hypothermic machine perfusion
HO	Heme oxygenase
HRQL	Health related quality of life
HTK	Histidine-tryptophan-ketoglutarate
IF	Immunofluorescent
IFN	Interferon
IGF	Insulin growth factor
INR	International normalized ratio
IRI	Ischaemia reperfusion injury
ITU	Intensive Therapy Unit
IU	International unit
IV	Intravenous
KCL	Potassium chloride
KO	Knockout
LAP	Latency associated peptide
LDH	Lactate dehydrogenase

LDLT	Living donor liver transplant
LFT	Liver function test
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
LT	Liver transplantation
MAFLD	Metabolic dysfunction-associated fatty liver disease
MAPK	Mitogen activated protein kinase
MELD	Model for end stag liver disease
MINORS	Methodological index for non-randomized studies
MMP	Matrix metalloproteinases
<i>MRNA</i>	Messenger RNA
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Non alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NK	Natural Killer
NKT	Natural Killer T cell
NMP	Normothermic machine perfusion
NQOI	NAD(P)H dehydrogenase [quinone] 1
<i>OLT</i>	Orthotopic liver transplant
OPTN	Organ Procurement and Transplantation Network
OR	Operating room
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
<i>pERK</i>	Protein Kinase RNA-Like ER Kinase
PNF	Primary non function
<i>PPAR</i>	Peroxisome proliferator-activated receptors
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
PVDF	Poly(vinylidene fluoride)

RCT	Randomised Controlled Trial
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SCS	Static cold storage
SD	Standard deviation
SEM	Standard error of mean
<i>SRTR</i>	Scientific Registry of Transplant Recipients
SYRCLE	Systematic Review Centre for Laboratory Animal Experimentation
TG	Triglyceride
TGF	Transforming growth factor
TPN	Total parenteral nutrition
UNOS	United Network for Organ Sharing
UW	University of Wisconsin solution
VEGF	Vascular endothelial growth factor
VITTAL	Viability Testing and Transplantation of Marginal Livers
WB	Western Blotting
WHO	World Health Organization
WIT	Warm ischaemia time
WUSM	Washington University School of Medicine

Summary

Liver transplantation is the only effective curative treatment for end-stage liver disease and modern-day practice has led to excellent survival outcomes. Ensuring access to suitable allografts remains crucial as patients who are unable to receive an organ face a dismal prognosis on the wait list. This challenge has been further complicated by dynamic changes to population health owing to the rise in metabolic conditions which have impacted the quality of the current donor pool and utility of potential allografts. Interventions to increase the donor pool have recently focused on increasing the use of extended criteria or marginal allografts that would otherwise be rejected while also reducing organ discard. The challenge in using marginal livers is in their inability to withstand ischaemia reperfusion injuries and regenerate after the post-operative period. Given the critical role of inflammatory and antioxidant pathways during the reperfusion process, defining the molecular mechanisms of allograft injury and marginal liver responses during this period may help identify therapeutic targets and salvage a proportion of discarded organs.

Upon reperfusion, a burst of reactive oxygen species (ROS) leads to the activation of several physiological processes involved in both tissue injury and repair. During this process, ROS further stimulate the NRF2 mediated pathway which has antioxidant, anti-inflammatory and regenerative functions. The pathway plays a key role in cellular defence and may have critical effects on liver allograft stress. The overall aim of this thesis was to assess the relevance of this axis in discarded and marginal liver allografts with a view to targeting the pathway, especially in fatty livers, to improve functionality.

NRF2 protein was measured in discarded human liver allografts and variable levels were observed. Increased expression of NRF2 was linked to favourable hepatic clinical parameters and less evidence of inflammatory injury. NRF2 levels could therefore become a useful indicator of marginal allograft selection in the clinical setting. Using an ex vivo normothermic machine preservation model, livers were exposed to six hours of perfusion and hepatic viability was investigated. Livers with higher levels of NRF2 consistently demonstrated superior functional profiles compared to livers with lower or absent NRF2 levels.

Steatotic livers demonstrated significantly lower NRF2 levels when compared to lean livers suggesting their increased susceptibility to injury during surgery. Interestingly, the results indicate that steatosis does not automatically imply marginality. To explore this further,

experiments were performed to identify a relative cut-off value for steatosis in which NRF2 may still function. Some, moderately steatotic and, on occasion, severely steatotic livers maintained intact NRF2 axes, emphasizing the potential of hepatic NRF2 activity as an important predictive marker of organ suitability.

Following on from the previous findings, NRF2 was then modulated in a fatty liver rodent model using a pharmacologic agent (Oltipraz) in combination with the benefits of ex vivo preservation. The results suggest that an NRF2 agonist can be used effectively to increase activity at a nuclear level allowing steatotic livers to resist significant reperfusion injury.

Evolving population demographics and the resurgence of metabolic syndromes have unequivocally impacted access to suitable allografts. Identifying which livers can be salvaged for clinical use may help bridge the gap between the donor and recipient pool. Encouragingly, the NRF2 pathway can offer insight into the inflammatory responses and regenerative potential of livers during the transplantation process. The findings from this work can help improve our current understanding of allograft injury and propose additional systems for all organ development and selection.

Lay abstract

Liver transplantation removes a diseased or damaged liver and replaces it with a healthy one from a donor. This is often the last remaining option when a person's liver is badly damaged because of disease, infection or cancer and cannot perform regular functions. Unlike kidney failure, there is no dialysis option or device to temporarily replace the liver's function in the body and liver transplantation is the only option. Although the operation is challenging and the patient who receives the liver transplant (recipient) will have to remain on lifelong immunosuppression medication to prevent the body from rejecting the liver, the prognosis can be excellent.

Healthy livers from donors are scarce and some patients can remain on the transplant wait list for a long time or die before they receive a liver. As a result, there is a large focus within the transplant community to increase the number of suitable livers that could be available for transplantation. The increasing prevalence of obesity and diabetes in the population means that there are now even fewer livers for donations. These livers have traditionally been rejected to transplant because they are more susceptible to injury during surgery. However, there is new scientific evidence that some of these livers, known as marginal livers, might function normally and can therefore be successfully transplanted into specially selected patients.

In order to study how a marginal liver might behave during transplant, this research has studied the role of a protein called NRF2 which is part of a pathway to help fight liver damage as part of the cell's defence system and to restore normal functions. We thought this pathway would be important in donor livers. Through several experiments, we found that NRF2 is present in variable amounts in different donor livers. We then found that livers with higher levels of NRF2 functioned better and had less injury than livers with lower levels. We also found that livers with a high amount of fat from obese donors have less NRF2 levels compared to livers from lean donors.

Traditionally, when a liver is removed from a donor, it is stored on ice until it reaches the selected recipient who receives the organ. Recently, a new device has been developed to keep the liver alive outside the body for some hours within a machine. The liver can be maintained at normal body temperatures of 37°C and blood, medications and fluids can be circulated through the system. This technology is known as machine perfusion and is designed to help improve the function of the liver. As part of our experiments, when livers with higher levels of NRF2 were studied on a normothermic machine model, these livers responded well on the machine and were more functional than livers with lower or absent NRF2 levels. Finally, using a rat model of fatty liver disease, we have shown that we could increase NRF2 levels in fatty livers during machine perfusion and this resulted in less liver injury.

We conclude that marginal livers could be better suited to transplantation if they have functioning levels of NRF2 which would help restore their function and offer extra protection during surgery. The ultimate goal of these studies is to contribute to the growing body of work on marginal allograft donations and improve the management of patients with liver failure by increasing the number of suitable donor livers. It is hoped that this would result in reduced waiting times on the liver transplant wait list and help save more lives.

Hypothesis and aims of the project

We hypothesize that the NRF2 axis influences allograft quality in liver transplantation and may provide a rational biomarker for allograft selection and therapeutic targeting. The overall objective of this thesis is to explore the role of NRF2 on the functionality of discarded or marginal liver allografts.

Specific aims

The specific aims of this thesis are to:

1. Determine the functional expression of NRF2 in discarded human liver samples and its impact on hepatic function and histopathology
2. Investigate NRF2 changes during normothermic machine perfusion
3. Assess the relationship between NRF2 and hepatic steatosis
4. Review current strategies using NRF2 analogues in the setting of liver transplantation
5. Examine the effects of pharmacologic NRF2 upregulation in steatotic samples in a murine ex vivo machine perfusion model

The value of this research

Liver transplantation is the only effective modality treating end stage liver disease. Initial experiences were disappointing in the 1950s and 1960s with many centers reporting unacceptable graft survival outcomes and patient mortality. Since then, major advances have been made in surgical technique, post-operative recovery and immunosuppression and transplantation has become widely accepted worldwide. The challenge now is in meeting the growing transplant waiting lists as the number of candidates added has increased remarkably making it difficult to match the same number in donors. At present, the liver donor pool cannot meet the needs of the patient population and a proportion of patients will never receive a liver, despite this being their only option for survival. Wait-list mortality is a powerful metric for assessing the effectiveness of transplantation by indicating the number of patients who will die waiting for a liver in transplant programs. This has not changed significantly over the last two decades. Consequently, increasing the number of actual donors and suitable liver allografts for transplant is an important topic.

Minimizing organ discard by reconsidering allografts that would have traditionally been discarded is one avenue worth considering and redefining the boundaries of allograft suitability is an underlying theme in this thesis. It has been determined that not all discarded allografts are permanently damaged and some may still remain functional. As such, the definition of extended criteria organ donors is an ever-evolving topic. Increasing the utility of these organs can potentially be achieved by minimizing the process of ischaemia reperfusion injuries to which they are more susceptible. This is challenged by a complex hepatic immune repertoire and understanding how different livers respond to injury can lead to the informed development of targeted therapies that could enrich the donor pool and guide allograft selection.

Based on the experiments of this thesis, hepatic NRF2 appears to be a promising target in enhancing allograft function and a greater understanding of the molecular profile of antioxidant pathways and ischaemia reperfusion injuries in marginal livers is required. This information can help to accurately identify the suitability of livers which would otherwise be discarded for transplantation by assessing their NRF2 yield and to rationally target livers in order to adequately restore mechanisms. This has the potential to improve patient outcomes and wellbeing by increasing the number of available livers to patients on the wait list while also avoiding the unnecessary waste of valuable resources.

Outputs

Publications

Ahmed, O., Robinson, M. W., & O'Farrelly, C. (2021). *Inflammatory processes in the liver: divergent roles in homeostasis and pathology*. Cellular & Molecular Immunology, 18(6), 1375-1386.

Ahmed, O., Xu, M., Zhou, F., Wein, A. N., Upadhyay, G. A., Ye, L., Wong, B., O'Farrelly, C., Chapman, W. C. (2022). *NRF2 assessment in discarded liver allografts: A role in allograft function and salvage*. American Journal of Transplantation, 22(1), 58-70.

Ahmed O, Doyle MBM. *Liver transplantation: expanding the donor and recipient pool*. Chin Clin Oncol 2021;10(1):6. doi: 10.21037/cco-20-212

Oral Presentations

The Royal College of Surgeons Annual Charter Meeting; The Presidents Session (finalist),

April 2022

*Expanding the Liver Donor Pool in an Era of Marginal Livers – the Role of Antioxidant
Defence Mechanisms*

Ahmed, O., Xu, M., Zhou, F., Wein, A. N., Upadhya, G. A., Ye, L., Wong, B., Yiing, L.,
O’Farrelly, C., Chapman, W. C.

Sir Peter Freyer Surgical Symposium, September 2021

*Evaluating Antioxidant Mechanisms in Discarded Liver Allografts: The Role of NRF2 in
Organ Salvage*

Plenary session (winner)

Ahmed, O., Xu, M., Zhou, F., Wein, A. N., Upadhya, G. A., Ye, L., Wong, B., Yiing, L.,
O’Farrelly, C., Chapman, W. C.

XXX Waterford Surgical October Meeting 2021

*The Role of NRF2 in Fatty Liver Allografts: Optimizing Allograft Potential for
Transplantation*

Ahmed, O., Xu, M., Zhou, F., Wein, A. N., Upadhya, G. A., Ye, L., Wong, B., Yiing, L.,
O’Farrelly, C., Chapman, W. C.

Owen H. Wangenstein Scientific Forum American College of Surgeons Clinical Congress

(Virtual) March 2020

*NRF2 Appears to Be Downregulated in Steatotic Compared with Lean Liver Allografts - A
Potential Role for Targeted Therapies*

Ola Ahmed, MD, Min Xu, MD, Fangyu Zhou, MD and William Cavanaugh. Chapman,

Poster Presentations

American Transplant Congress, June 2021 (virtual meeting)

NRF2 assessment in discarded liver allografts: A role in allograft function and salvage

American Association for the Study of Liver Diseases, November 2021 (virtual meeting)

The Liver Meeting

Interrogating the role of NRF2 in steatotic liver allografts: optimizing strategies for organ selection

The 31st Conference of the Asian Pacific Association for the Study of the Liver March

2022

Optimizing Fatty Liver Allograft Potential for Transplantation Using the NRF2 Axis

Chapter 1 - Thesis introduction

1.1 Anatomical structure of the liver

The liver is the largest organ in the human body and is principally involved in several biosynthetic, biodegradative, metabolic and physiologic pathways[1]. It consists of distinct functional components that are largely defined by its unique dual blood supply, receiving two-thirds of its input from the portal vein and the remaining one-third from the hepatic artery[2]. Macroscopically, the liver is divided into a right and left lobe by the falciform ligament, with the right lobe further subdivided into two smaller caudate and quadrate lobes posteriorly and inferiorly (Figure 1.1).

The liver is then further subdivided into eight independent functional units or segments as described by Couinaud in 1957, each segment with its own dual vascular, biliary and lymphatic drainage[3]. In this classification, the middle hepatic vein divides the liver into right and left lobes, the right hepatic vein divides the lobe into anterior and posterior segments and the left hepatic vein into a left medial and lateral section. The portal vein forms a horizontal plane further subdividing the liver into superior and inferior segments. This classification allows for individual segmentectomies in liver surgery while also maintaining the integrity of surrounding segments.

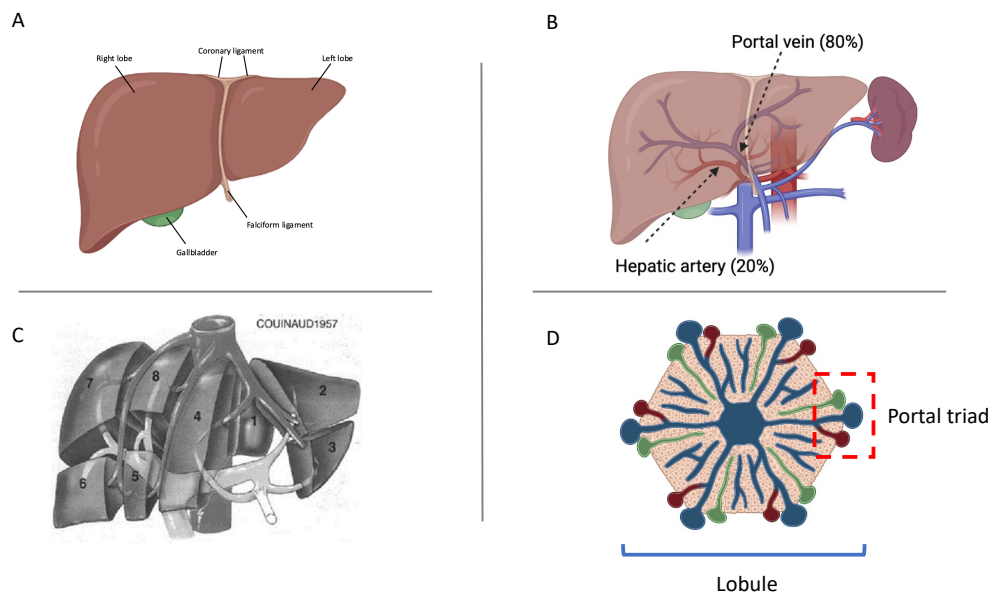


Figure 1. 1 *Anatomical structure of the human liver.* **(A)** The liver consists of 2 main lobes, a right and left lobe, that are divided by the falciform ligament. This is a sickle shaped ligament that attaches the anterior surface of the liver to the anterior abdominal wall. Two further lobes arise from the right lobe and are located on the visceral surface of the liver, the caudate and quadrate lobes. On the superior surface of the liver, the coronary ligament attaches the superior surface of the liver to the inferior surface of the diaphragm. **(B)** The liver has a unique dual blood supply. The hepatic artery proper arises from the celiac trunk to supply the liver with 20% of its blood supply in the form of oxygenated arterial blood. The portal vein is the dominant blood supply to the liver providing 80% of its blood input and supplying deoxygenated blood. **(C)** The Couinaud classification further subdivides the liver into 8 functionally independent segments each with its own vascular inflow, outflow and biliary drainage. **(D)** Microscopically, the liver appears as structural units called lobules. Each lobule is hexagonal shaped with the portal triad of an arteriole, a venule and a bile duct at its periphery.

1.2 Liver failure

1.2.1 Acute on chronic liver failure

Acute liver failure (ALF), first described in 1970, is characterized by acute liver injury, jaundice, coagulopathy (INR > 1.5) and hepatic encephalopathy without the presence of preexisting liver disease [4, 5]. In 2012, a systematic review identified 41 different definitions for ALF, the main differentiating factor being the defined interval between the onset of liver disease and hepatic encephalopathy [6]. Three specific time frames are applied to categorize and distinguish the different forms of ALF; hyperacute (< 7 days), acute (7 to 21 days) or subacute (21 days to 26 weeks). The European Association for the Study of the Liver (EASL) characterizes chronic liver disease as hepatic impairment beyond 28 weeks and before the onset of encephalopathy [7]. The most recent definition in hepatic failure nomenclature is “acute-on-chronic” liver failure which is characterized by an acute deterioration in liver function in patients with underlying chronic liver disease [8, 9]. Liver cirrhosis is characterized by progressive, diffuse and irreversible fibrosis that completely disrupts the parenchymal architecture [10].

Depending on disease severity and timing, ALF and CLF can present with a variety of non-specific syndromes and liver failure is oftentimes referred to as a “silent disease”[10].

Clinical manifestations may vary from mild right upper quadrant pain, fatigue, nausea and anorexia to jaundice and progressive mental changes as the disease evolves.

Liver failure can be diagnosed and confirmed using a variety of mediums. Following a thorough history and examination, initial testing includes serum liver function tests. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are released when hepatocytes are damaged or killed. Although plasma membrane damage and protein leakage from oncotic necrosis are likely the most common reasons for elevated transaminase levels, other mechanisms could also be involved [11, 12]. Microscopic studies have shown membrane blebs containing cytosolic components forming within hepatocytes and rupturing without causing widespread cell death and still resulting in a rise in transaminases [13-15]. Alkaline phosphatase (ALP) is released when the biliary epithelium is injured such as in instances of biliary obstruction or cholangitis. γ -Glutamyltransferase (GGT) is expressed by both hepatocytes and the biliary epithelium and can be elevated in all

conditions impacting liver function[2]. Serum markers of liver function may vary depending on whether acute or chronic liver failure ensues (Table 1.1).

Table 1. 1 *Serum liver testing in acute and chronic liver failure* [2]

	Acute	Chronic
Albumin	-	+++
Prothrombin time	+++	+++
Bilirubin	+	+++
Lactate	++	-
Glucose requirement	++	-
Ammonia	+	+

The cause of liver failure influences the management approach and prognosis and for this reason every effort is made to establish the correct underlying pathology. A series of standard tests are considered in every patient and if the diagnosis still remains unclear, a liver biopsy is performed provided there are no absolute contraindications.

1.2.2 Liver Failure Scoring systems

1.2.2.1 Child-Pugh Score

Several liver failure scoring systems have been proposed to assess the severity of disease, determine the likelihood of developing complications of cirrhosis and offer prognostic information[16, 17]. The Child- Pugh score was first developed in 1973 and is currently used to assess prognosis and survival in patients with chronic liver disease [18]. Five variables are incorporated into the score including serum albumin, serum bilirubin, ascites, encephalopathy and prothrombin time and the score ranges from 5 – 15. This classification system categorizes patients into 3 groups correlating with their respective scores: class A (well compensated), class B (significant functional compromise) and class C (decompensated cirrhosis). One-year survival rates for patients with class A, B and C Child-Pugh are 100%,

80% and 45%, respectively[17]. The scoring system has also been validated to predict mortality risks in abdominal operations [19]. Following abdominal surgery, mortality rates are 10%, 30% and 70% to 80% for class A, B and C [20].

Table 1. 2 *Child-Pugh classification for liver disease*[18].

Child-Pugh Score			
Variable	1 point	2 points	3 points
Total Bilirubin ($\mu\text{mol/L}$)	<34.2	34.2 – 51.3	>51.3
Serum albumin (g/L)	>35	28 – 35	<28
Prothrombin time			
Seconds over control	<4	4 – 6	>6
INR	<1.7	1.7 – 2.3	>2.3
Encephalopathy	None	Grade I – II	Grade III - IV
Ascites	Absent	Mild	Moderate - Severe
	Class A	Class B	Class C
Total points	5-6	7-9	10-15
1-year survival	100%	80%	45%

1.2.2.2 King's College Criteria

The King's College Criteria were developed in 1989 by exploring data of 588 patients with ALF [21]. It was initially applied to determine prognosis in paracetamol-induced fulminant hepatic failure and is used to predict survival outcomes for alternative etiologies of hepatic failure. Additionally, the framework can be an adjunct in prediction tools when proposing candidacy for liver transplantation [22]. Positive predictive values for paracetamol and non-paracetamol induced ALF are 84% and 98%, respectively. Negative predictive values are 86% and 82% for paracetamol and non-paracetamol induced ALF [22].

Table 1. 3 *King's College Criteria to determine which patients with fulminant liver disease should be referred for transplantation* [21]

Paracetamol –induced disease
Arterial pH <7.3 (irrespective of the grade of encephalopathy)
<i>OR</i>
Grade III or IV encephalopathy <i>AND</i>
Prothrombin time >100 seconds <i>AND</i>
Serum creatinine >3.4mg/dL (301 µmol/L)
All other causes of acute liver failure
Prothrombin time >100 seconds (irrespective of the grade of encephalopathy)
<i>OR</i>
Any three of the following variables (irrespective of the grade of encephalopathy)
○ Age <10 years or >40 years ○ Etiology: non-A, non-B hepatitis, halothane hepatitis, idiosyncratic drug reactions ○ Duration of jaundice before onset of encephalopathy >7 days ○ Prothrombin time >50 seconds ○ Serum bilirubin >18 mg/dL (308 µmol/L)

1.3 Liver transplantation surgery

In 1967, Thomas Starzl attempted the first human liver transplantation (LT) in a 3-year-old child with biliary atresia who unfortunately succumbed to the procedure and died in the operating room from uncontrollable hemorrhage [23]. However, just a few years later, Starzl successfully performed an LT in eight children, all of whom survived the operation, and half were alive at 1-year post-operatively [24, 25]. Since then, LT has become an exemplar model of complex surgery and the only curative option for patients with end stage liver disease (Figure 1.2). The United States (USA) currently has the highest transplant volume, performing over 8000 procedures in 2019 alone and witnessing a growing wait-list annually [26]. In the UK, over 800 liver transplants are currently performed annually in 7 designated units [27, 28]. In Ireland, liver transplant numbers vary between 44 and 62 cases per annum and are only performed in one national liver transplant unit, St Vincents Hospital Dublin. Although hepatitis C remains the most common indication for LT worldwide, alcohol associated liver disease (ALD) is the most common indication in the US, UK and Ireland, accounting for 23% - 33% of transplant recipients [29]. The proportion of candidates listed with a primary diagnosis of non-alcoholic fatty liver disease (NAFLD) has also increased steadily in the past decade. This appears to parallel with the commensurate rise in population obesity and while it currently accounts for approximately 10% of LTs in the US, NAFLD has been projected to surpass ALD in the coming decade as the leading indication for LT [30]. For now, graft and patient survival outcomes following LT continue to improve in contrast to the early innovative days of LT during Starzl's time. One- and 5-year patient survival exceeds 85% and 80%, respectively, and LT has been widely adopted by over 80 programmes worldwide [26, 31].

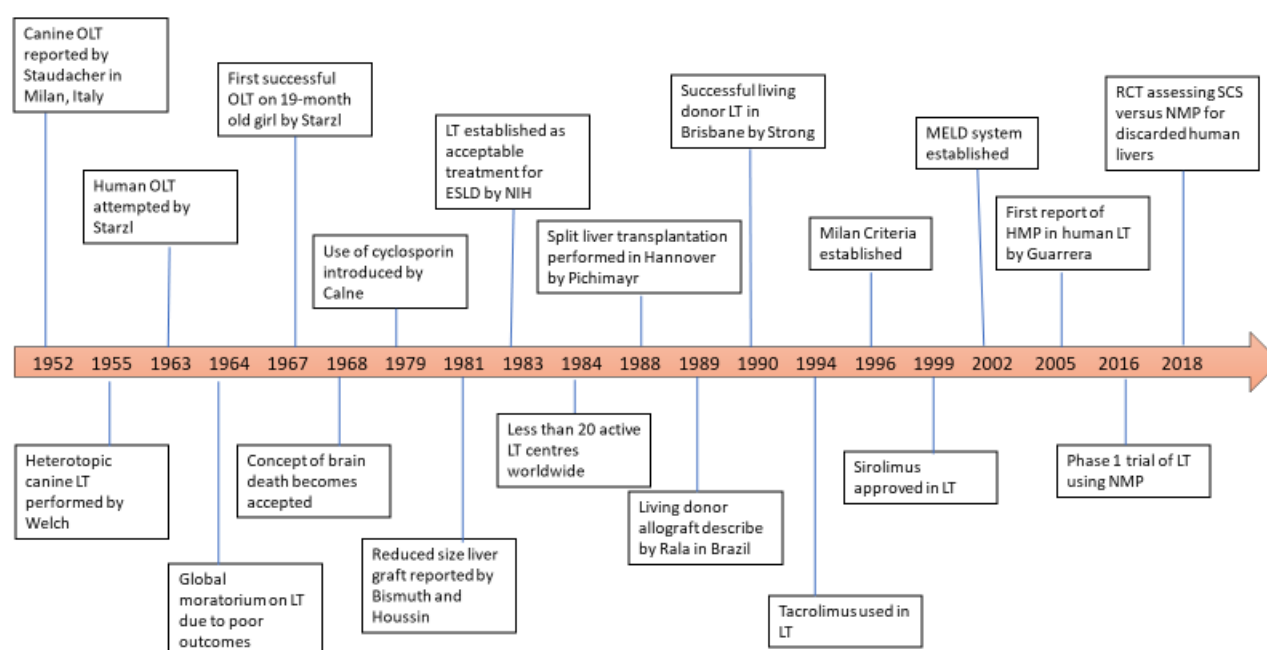


Figure 1. 2 *Important milestones in liver transplantation*[31].

1.3.1 Organ shortages in liver transplantation

At current rates, less than 10% of global organ transplantation needs are met due to challenges in identifying suitable allografts for solid organ transplantation[32]. The factors underpinning this deficit are largely related to geographic variation in disease prevalence which can minimize the suitability of potential organ donors and access to treatment [33-36]. Compounding these issues are the alarming projections of reduced organ quality. In the next two decades, donor liver utilization is expected to fall to 44% if current practices remain constant, adding further strain to the donor population [37]. This has large implications for the liver candidate pool and wait-list mortality varies between 10 – 30% for potential recipients[38, 39]. Unsurprisingly, these forecasting models parallel the increasing prevalence of metabolic syndrome, viral hepatitis, alcoholic cirrhosis and the full spectrum of NAFLD [33, 40]. Attention now is becoming focused on the discrepancy between organ supply and demand as the number of new entrants onto the liver transplant waiting list continues to rise [26]. Many efforts have been made to expand the donor pool including restructuring surgical techniques to accommodate split liver grafts and living donors as well

as the consideration of donation after circulatory death (DCD) organs, steatotic allografts and allografts from older donors [41].

1.3.2 Organ quality and extended criteria donor organs

The aging population, longer life expectancy rates and the rising incidence of metabolic conditions such as obesity, diabetes and fatty liver disease are all important factors contributing to poor donor quality. In this setting, increasing the utility of “marginal” livers and reducing organ discard, currently at 9%, may alleviate some of the pressures on the LT wait-list[26]. Amid the increasing organ shortages, the transplant community has proposed meticulous organ salvage strategies and the utility of extended criteria donor (ECD) organs, previously considered less than optimal or “marginal”, which do not meet the traditional criteria for organ donation [42, 43]. There is no universally accepted definition for what constitutes an ECD or marginal liver, however, some described and frequently cited characteristics are advanced donor age, donation after cardiac death (DCD), hepatic steatosis, split liver transplantation (SLT), and donors with an infectious risk or previous malignancy (Figure 1.3) [43-45]. A major discrepancy in the definitions is in the age cut-off for what could be considered as an “older donor” and this ranges from 50 to 70 years [46].

Criteria for marginal liver donors: one or more of the following points:

- Donor age > 65 years
- ICU stay with ventilation > 7 days
- BMI > 30Kg/m²
- Steatotic liver > 40%
- Serum sodium > 165 mmol/l
- SGPT > 105 U/l
- SGOT > 90 U/l
- Serum bilirubin > 3 mg/dl
- Donation after cardiocirculatory death
- Donation after euthanasia

Figure 1. 3 *Definitions for marginal donors according to Eurotransplant.*

While these organs were previously avoided due to the fear of primary non-function (PNF) or delayed graft function (DGF), a select subset are now increasingly used with the goal of improving access to transplantable organs [45]. Their use in LT remains limited and marginal allografts are most commonly used in high volume transplant centers [47-50]. Current evidence suggests that marginal livers account for 35% of the current liver donor pool [46]. Thus, although more patients are referred for transplant, the number of suitable grafts from deceased donors has increased at a much slower rate. In the USA, DCD livers account for only 8.5% of transplanted livers [51] but this is slightly higher in the UK with reports of DCD livers accounting for 14% - 20% of liver transplants [52, 53]. Up to 30% of retrieved DCD livers are discarded and many DCD organs are declined without being retrieved [54, 55]. In Ireland, DCD livers are still not part of the donor pool. The underlying concern is the graft's susceptibility to ischaemia/reperfusion injuries (IRI) during the transplantation process, adding to graft dysfunction and poor liver regeneration [56]. Despite this risk, ECDs have played an important role in expanding the donor liver pool with compelling evidence demonstrating their ability to reduce wait list mortality and, in some instances, exhibit recipient outcomes comparable to standard liver donations [57, 58].

1.3.3 Ex vivo machine perfusion

Allograft machine preservation has been recognized as one of the most significant contributions to liver transplantation in the past two decades [59]. Machine perfusion technology has been used in kidney transplantation since the late 1960s and was initially described using hypothermic temperature storage inducing a metabolic "rest" in cellular function[60]. Similar technology was then applied to liver transplantation and the arrival of ex-vivo machine perfusion techniques is beginning to change the landscape of how "marginal" livers are utilized with the potential to fully expand the donor pool and reduce liver discard. In all cases, machine perfusion is applied ex vivo after liver procurement and can offer a number of advantages to the organ donation and transplantation process such as conditioning the liver prior to implantation and avoiding irreversible injury while also offering an opportunity to test organ quality before transplant (Figure 1.4) [59].

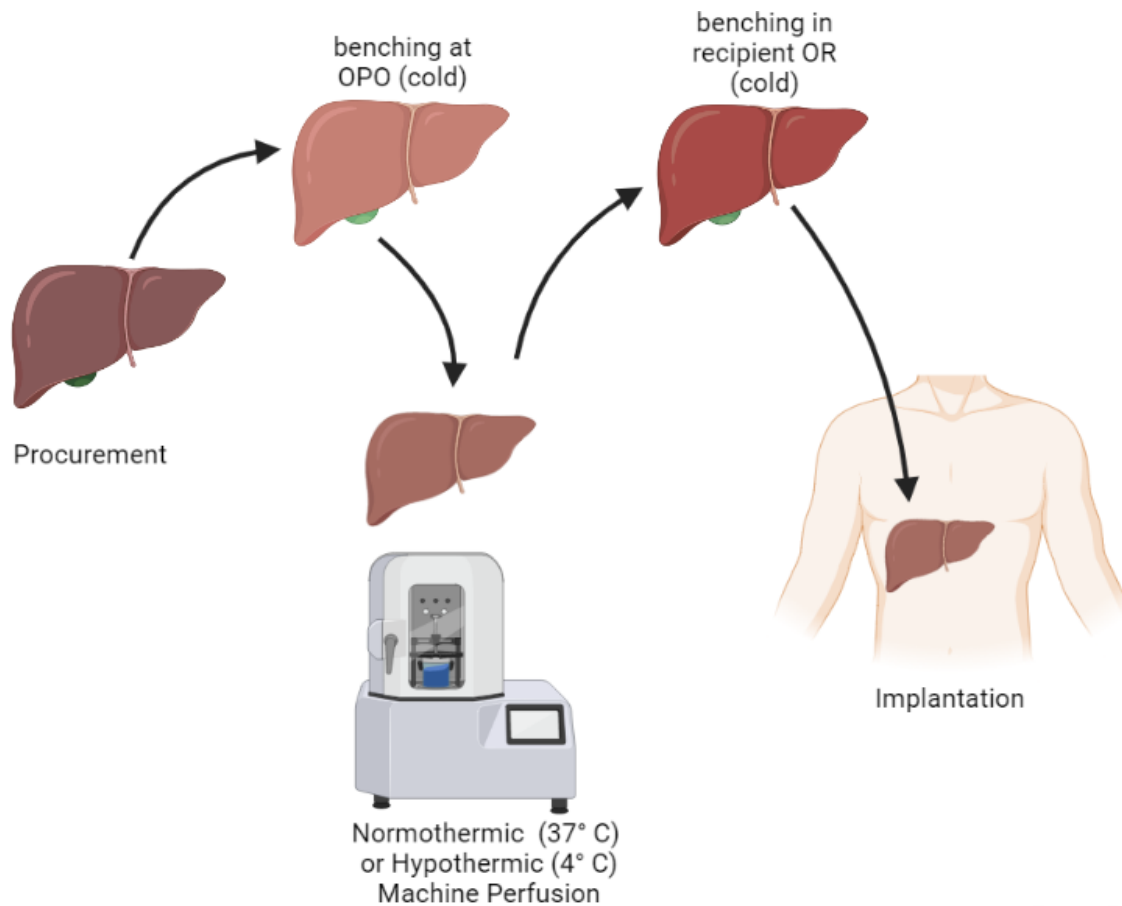


Figure 1. 4 *Ex vivo machine preservation during liver transplantation*. Following procurement from the deceased donor, the organ is “benched” and the blood supply is cannulated. The allograft is then placed onto the ex vivo machine device at hypo- or normothermic temperatures for 4 – 24 hours per institutional protocols. In the case of NMP, viability assessments are performed before the liver is taken off the machine to measure its function. After machine perfusion, the liver is removed from the machine and implanted into the selected recipient.

During standard cold storage (SCS), hypothermia decelerates cellular metabolism and reduces allograft oxygen demands[61]. Cellular metabolism does not cease completely and the hepatocyte relies on anaerobic metabolism at a lower rate leading to the depletion of adenosine triphosphate (ATP) stores [62]. Significant depletion in ATP levels causes ionic dysregulation due to the inability of the membranous sodium-potassium ($\text{Na}^+\text{-K}^+$) ATPases to function appropriately, leading to calcium accumulation and a low pH [63, 64]. There is also evidence to suggest that ischaemia increases cAMP levels, stimulating glycolysis and the subsequent accumulation of hexose-6-phosphates and lactate [61, 65]. Hypothermic

preservation (2°C to 10°C) utilizes dissolved oxygen in a blood-free perfusate to also slow down cellular metabolism. The added advantage of HMP over SCS is the ability of HMP to deliver metabolic substrates to the liver and also wash out metabolic waste products [66]. Additionally, HMP has been shown to lessen some of the hepatocellular structural changes that occur during SCS and reduce the number of CD68+ macrophages in livers [67].

In contrast, normothermic machine perfusion (NMP) aims to simulate *in vivo* conditions using oxygenated blood-based perfusate at physiologic temperatures (37 °C) and hepatic flow rates [68]. Ischaemia is prevented by perfusing the organ with an oxygen rich medium while simultaneously delivering metabolic substrates and removing waste products to allow for continuous ATP production [69]. As such, NMP can sustain full metabolism. Additionally, NMP has been shown to upregulate cytoprotective proteins such as HO-1 and members of the heat shock family [70, 71].

Both hypothermic (HMP) and normothermic (NMP) machine perfusion technologies have shown promising results in minimising injury to DCD grafts and steatotic livers [72, 73]. Schlegel and colleagues provided 5-year outcome data on patients receiving DCD liver transplants following treatment with hypothermic oxygen perfusion prior to transplantation. Outcomes of treated DCD livers were similar to livers from standard brain-dead donors and superior to untreated DCDs with significantly less graft loss events [72]. A recent study also reported encouraging results in transplanting previously rejected liver allografts following the application of NMP [74]. The unique advantage of NMP over HMP is the ability to perform timely viability and metabolic assessments on the allograft during preservation that could assist with clinical decision making, recipient selection and logistics in theatre preparation time [75]. Another advantage of NMP over HMP is the ability to extend preservation time since a physiological environment is being replicated and theoretically the organ would not have to adhere to strict and specific cold ischaemia times [69]. Additionally, this would optimize recipient selection, tissue matching and also facilitate long distance sharing of organs. From a therapeutic perspective, a normothermic environment could allow for the addition of drug compounds that are more effective when administered in a physiologic environment and can be delivered directly to the organ on the machine and reduce systemic loss [69, 76]. Strong evidence suggests that the expression of

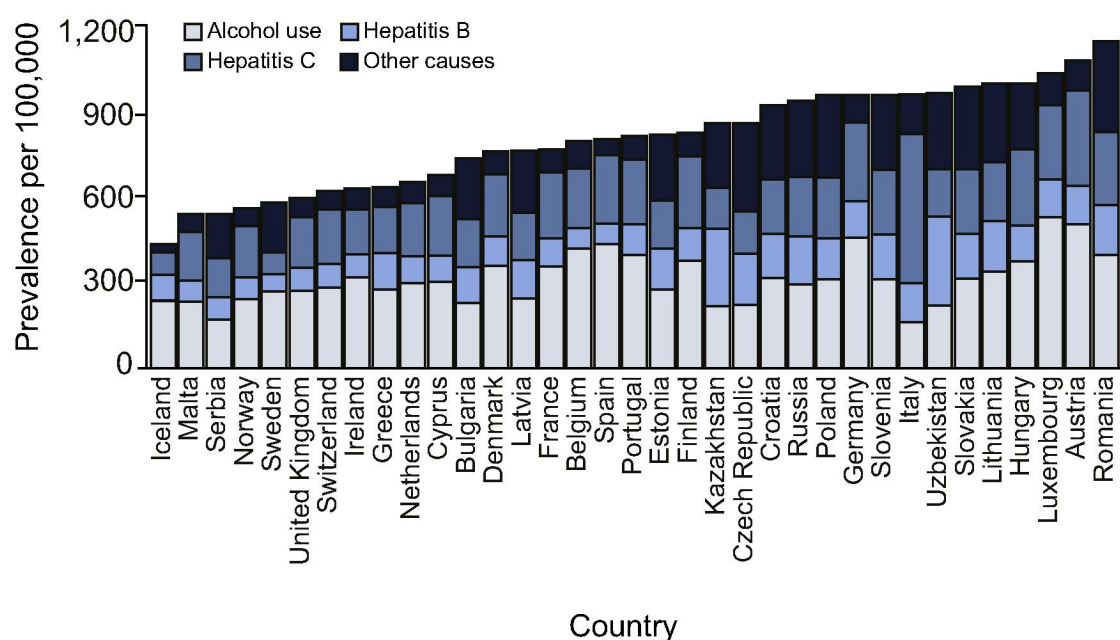
genes and the induction of new protein synthesis are active cellular processes that are negatively affected in low temperatures[76]. This innovation may salvage various extended criteria allografts and modify their risk for routine use, ultimately expanding the donor pool.

Table 1. 4 *Differences between normo- and hypothermic ex vivo machine perfusion*[77].

	Normothermic Machine Perfusion	Hypothermic Machine Perfusion
Temperature	35–38 °C	4–10 °C
Pathophysiology	Mimics in-vivo environment Continued cellular metabolism Prevents cold ischaemic injury Functioning liver during preservation	Improved cold storage Reduced cellular metabolism Reduces ROS release & inflammation Non-functioning liver during preservation
Perfusate	Continuous oxygen and nutrient delivery. Usually blood.	Non-specific, +/- Oxygen
Benefits	Allows for viability assessment and metabolic functioning during preservation	No option to determine liver viability

1.4 The Public Health Burden of Liver Disease

Liver disease is a key and critical public health burden, responsible for approximately 2 million deaths per year worldwide [32]. Internationally, cirrhosis ranks 4th among chronic diseases in terms of life years, preceded only by ischaemic heart disease, cerebrovascular disease and chronic obstructive pulmonary disease [78]. Global population data suggests a 500% increase in liver disease incidence. Recent epidemiological data, supported by the European Association for the Study of the Liver (EASL) HEPAHEALTH Steering Committee, reported concerning rates in Europe which appears to now account for the largest burden of liver disorders worldwide [79-81]. In Europe, the current age adjusted prevalence of liver disease ranges from 447 to 1,100 cases per 100,00 (Figure 1.5) [79].



Source: Global Burden of Disease database

Figure 1. 5 Age standardized incidence of liver disease by aetiology of countries included in the HEPAHEALTH Project[79]. The median prevalence of chronic liver disease is 833 cases per 100,000.

Accurate population statistics are often difficult to access and interpret due to sparse mortality data from many regions where liver disease is highly prevalent, such as Africa and South East Asia. Additionally, the lack of standardized definitions for certain conditions and an inherent selection bias stemming from reports that mainly originate from large tertiary

centres likely contributes to a significant under-reporting of cases. Despite these caveats, there is clear evidence from population models that acute and chronic liver disease is widely prevalent and leads to significant morbidity and mortality. The top four leading causes of liver-disease related death globally are hepatitis C (700,000 deaths annually), hepatitis B (250,000 deaths annually), alcohol related liver disease (500,000 deaths annually) and non-alcoholic fatty liver disease (250,000 deaths annually) [78]. Among these, the prevalence of NAFLD and NASH is projected to increase rapidly as a direct consequence of rising population obesity, becoming an important cause for liver impairment and organ failure [82, 83]. Alarming, a recent publication from the University of Bristol aimed to identify the prevalence of steatosis and fibrosis in young adults using transient elastography (FibroScan) and controlled attenuation parameter scores [84]. Out of 4021 participants with a mean age of 24 years, over 20% of young persons had hepatosteatosis and 10% were categorized as severe steatosis. Additionally, 1 in 40 young persons had hepatic fibrosis, which was linked to persons with unhealthy drinking patterns and established steatosis. With this trajectory, it appears inevitable that liver diseases will significantly impact the health of the future adult population if stringent public health measures are not in place.

Hepatic diseases also pose a tremendous impediment to health-related quality of life (HRQL), work productivity and economic structures [85-87]. In the US, individuals with liver disease were less likely to be employed than their healthy counterparts, lost more work and generated greater healthcare expenses. Although there is a gap in healthcare cost data, the direct cost of chronic liver disease in the US was estimated to be \$2.5 billion in 2004 [88]. The economic burden of liver disorders has further increased in line with the rising prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide. The annual direct medical costs of patients with NAFLD is estimated at \$103 billion annually in the US and a combined €35 billion in Europe (Germany, France, Italy, United Kingdom) [89]. Cumulative increases in the cost of managing liver disease related complications carries a 10-year economic burden of \$908 billion [90]

1.4.1 Epidemiology of Obesity and the Fatty Liver Disease

Obesity is defined as abnormal or excessive body fat. The World Health Organization (WHO) considers body mass index (BMI) as an accurate marker of increased weight and, for adults, a BMI greater than or equal to 30Kg/m² is considered obese [91].

Table 1. 5 *Body mass index as determined by the World Health Organization*

	BMI (Kg/m ²)
Underweight	< 18.5
Normal weight	18.5 – 25
Overweight	25 – 30
Obese class I	30- 35
Obese class II	35 – 40
Obese class III	>40

The global prevalence of obesity has increased substantially ranging between 11% - 15% among adult men and women and 6%-8% among girls and boys in 2016 [92]. Prevalence continues to accelerate and in a global analysis of trends in 195 countries in 2015, 107.7 million children and 603.7 million adults were classified as obese, tripling since 1975 and doubling since 1980 [93, 94]. In the United States (US), a recent nationwide analysis interrogating 20 years of data projected that by the year 2030, 1 in 2 US adults will be obese and 1 in 4 will be severely obese [95]. In European population data, over 1 in 5 adults are considered obese with staggering increases in the past 3 decades [96].

Currently in Ireland, the obesity rate approaches 25.3% of the entire population and 36% of the Irish population over the age of 50 years are obese [94, 97]. A pooled population based analysis of 200 countries in 2016 reported that the incidence and prevalence of obesity in Ireland is increasing more rapidly than in other European countries and epidemiological projections have predicted that Ireland will be the most obese country in Europe by 2025 [98]. In addition to reducing a person's lifespan, obesity confers significant disability, calculated at more than 36 million disability-adjusted life years in 2004, and numerous health risks such as, cardiovascular disease and cancer (Figure 1.6) [99]. The economic

burden of obesity is also vast, accounting for 0.7% - 2.8% of the total healthcare costs and obese persons are estimated to have 30% higher medical costs than normal weight individuals [100].

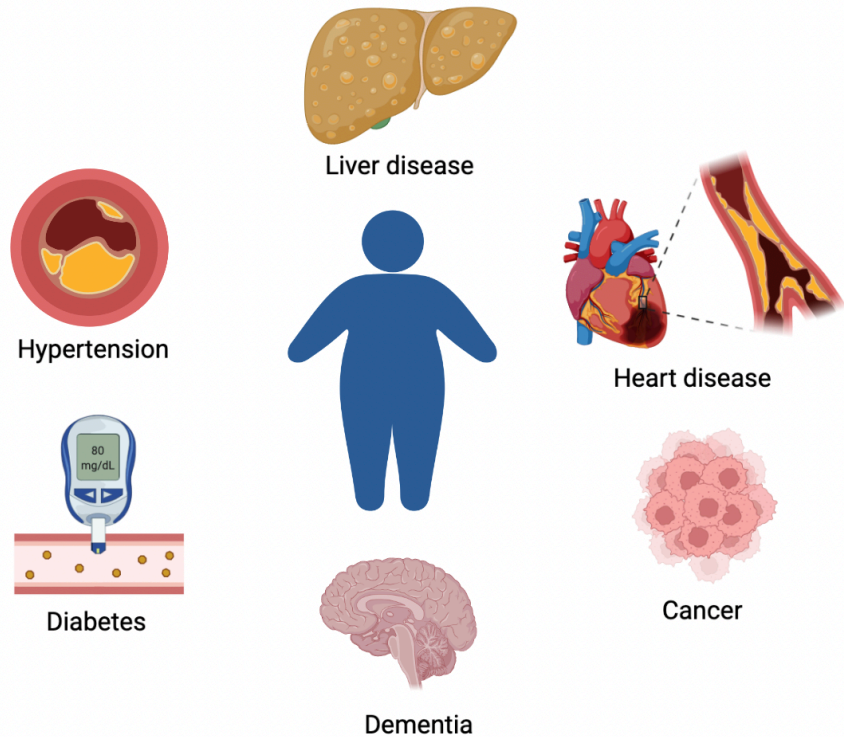


Figure 1. 6 *Obesity and the interlinked medical conditions.* Obesity is defined as a body mass index greater than or equal to 30kg/m^2 . The disease itself is a significant risk factor and contributor to morbidity and mortality from several chronic conditions such as cardiovascular disease, type 2 diabetes, cancers, dementia and liver disease.

1.4.2 Metabolic Health

The exponential rise in global obesity has also led to the increased prevalence of several metabolic conditions including diabetes, hypercholesterolemia and fatty liver diseases [101, 102]. The aptly named “metabolic syndrome” refers to the cluster of metabolic factors including obesity, hypertriglyceridemia, hypertension and insulin resistance. Remarkably, not all severely obese individuals manifest metabolic syndromes and 10- 25% are considered “metabolically healthy” [103, 104]. Such individuals are defined as having a BMI $\geq 30\text{ kg/m}^2$ without the presence of type 2 diabetes, dyslipidemia or hypertension [105].

Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the most common cause of chronic liver disease, affecting up to 30% of adults in the developed world. The increase strongly parallels the commensurate rise in global obesity and occurs in up to 90% of obese individuals, respectively [106-108]. The increasing prevalence in NAFLD also mirrors that of other closely related metabolic diseases and is largely driven by unhealthy lifestyles and diets [109]. NAFLD results from the pathological deposition of fat within liver cells in the absence of alcohol consumption [110]. This includes a spectrum of diseases ranging from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH) with or without fibrosis, becoming the most common liver disease in the world and the second leading indication for LT [108, 111]. Recently, a proposal has been made to modify the definition of NAFLD to *metabolic dysfunction-associated fatty liver disease* (MAFLD) to represent the hepatic manifestations of a multisystem metabolic dysregulation [112]. As with metabolic syndrome, a diagnosis of MAFLD is based on histological, radiological or serum confirmation of hepatic steatosis in addition to obesity, type 2 diabetes or at least 2 metabolic risk abnormalities [112].

At present, liver transplantation remains the only effective cure when irreversible liver injury occurs. Given the high prevalence of chronic liver disease, the impending population health crisis as a result of worsening metabolic diseases and the substantial economic health burden of cirrhosis, access to treatment needs to be improved. As such, increasing the availability of liver allografts should be a public health focus.

1.5 The immune cell repertoire and liver damage

The liver is an immunologically complex organ that functions as a physiological bridge between gut-derived molecules and the systemic circulation. Approximately 80% of the liver's vascular supply is provided by the portal venous system, resulting in a low-pressure blood system with large amounts of bacterial products and other foreign molecules being presented to the immune system. Consequently, the liver must tolerate these potentially immunogenic or inflammatory foreign molecules while maintaining constant immunosurveillance for infectious pathogens and liver malignancies. The hepatic microstructure is composed of an extensive repertoire of immune cells, embedded in a meshwork of liver sinusoids through which hepatic venous blood passes and mixes with oxygen-rich arterial blood from the hepatic artery. These sinusoids are lined by specialized liver sinusoidal endothelial cells (LSEC) that contain numerous fenestrations, allowing molecules to freely diffuse through to underlying hepatocytes, which perform the central metabolic functions of the liver [113] (Figure 1.7). This alignment facilitates the rapid exchange of blood-borne molecules and metabolic processing in the liver as well as extensive interactions with local and systemic immune populations.

Hepatic homeostasis relies on effective regulation of the intimate interactions between resident and infiltrating immune cells, and non-hematopoietic hepatic cells. Resident immune cells populate the liver sinusoids and the sub-endothelial compartment space of Dissé, located between hepatocytes and LSECs, and constitute a diverse cell population including professional antigen presenting cells (APC), myeloid cells and specialized innate and adaptive lymphoid cell populations [113]. Many hepatic immune populations play vital roles in homeostasis, tissue repair, maintaining immune tolerance and modulating liver inflammation.

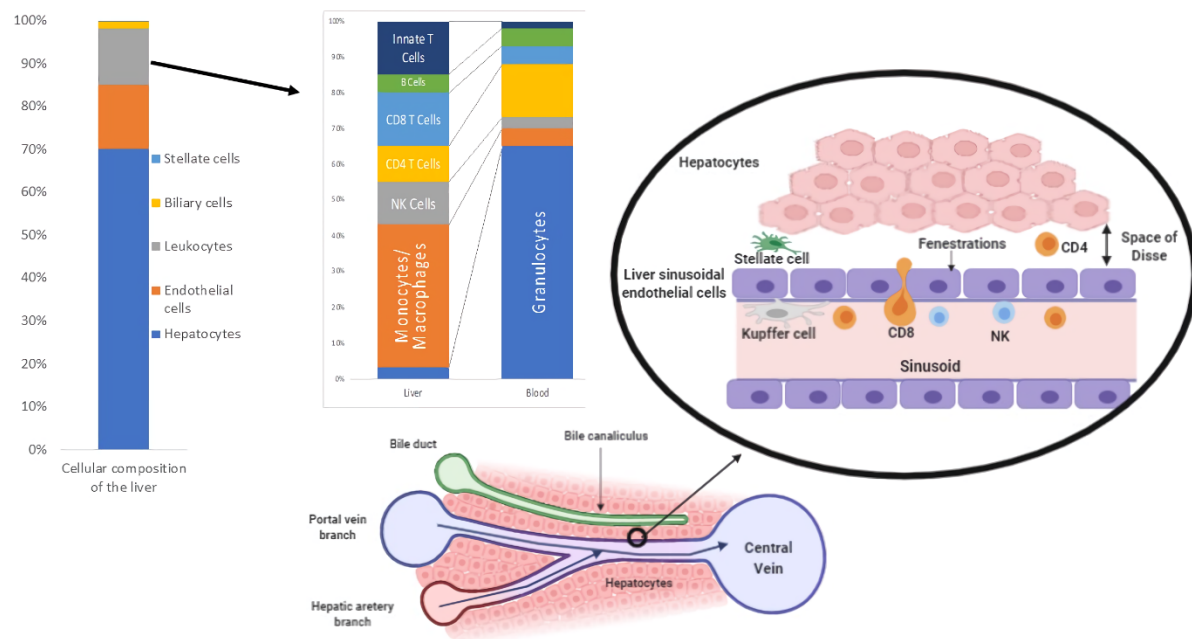


Figure 1.7 *The cellular composition of the liver.* Venous blood from the gut mixes with oxygenated arterial blood and drains through the sinusoids passing through plates of hepatocytes to the central vein. Hepatocytes secrete bile into the canaliculi and these secretions ultimately flow into the bile ducts. Sinusoids are lined by specialized, fenestrated liver sinusoidal endothelial cells allowing blood to pass through the space of Disse to the underlying hepatocytes. Within the blood space, Kupffer cells are adherent to the endothelial wall while resident lymphoid and myeloid immune cell populations are found around the portal tract and throughout the parenchyma [114].

1.5.1 Hepatic Immune Cell Repertoires

Hepatic immune cell populations have diverse roles in regulating inflammation and immune responses. Kupffer cells (KC), accounting for 80-90% of the total body population of fixed tissue macrophages and almost a third of the non-parenchymal cell populations in the liver, play a central role [115]. These liver-specific myeloid cells play a critical role in the recognition of blood-borne pathogens and their clearance of invading microbes [116, 117]. They have key roles in identifying, phagocytosing and eliminating 'foreign' or 'dangerous' antigens with their ability to scan using PRRs, complement receptors and Fc receptors. Consistent with their macrophage-like functions, KCs respond with increased phagocytic activity, however in contrast to other myeloid populations, they produce pro-inflammatory

and anti-inflammatory factors, illustrating their dichotomous role in tolerogenic defence and inflammation [118].

Dendritic cells (DC) are also important inducers of tolerance in the liver, although also capable of antigen presentation and type 1 interferon production [119-121]. They are categorized into myeloid (mDC) and plasmacytoid (pDC) subtypes and are primarily located around the central veins and portal tracts [122]. In physiological conditions, DCs are described as being phenotypically immature and tolerogenic and are thought to play an important role in tolerogenicity post-transplantation [123], however, proinflammatory DCs develop in response to liver injury, stimulating cytokine secretion and other T-cell responses [124]. There is evidence of participation in anti-inflammatory activity, particularly in the case of ischaemia reperfusion injury (IRI), indicating their dual immunosuppressive and pro-inflammatory roles [125].

Classical neutrophils are rarely detected in the healthy liver. However, neutrophil subsets with immunoregulatory properties, also referred to as type 2 neutrophils (N2), have been found amongst tumor-associated neutrophils (TANs) in hepatocellular carcinoma [126]. Such neutrophil subsets share phenotypic and functional features with mature low density neutrophils or granulocyte-like-myeloid-derived suppressor cells (MDSC) [127], which are known to suppress T-cell activation and, in the context of cancer, promote cancer growth [128, 129]. MDSC expansion has also been described in non-alcoholic fatty liver disease (NAFLD) [130].

Classic T-lymphocytes account for up to 50% of lymphocytes in the liver with B-cells comprising only 5% of the hepatic lymphocyte population [131]. While CD8⁺ T cells usually outnumber CD4⁺ T cells in a two-to-one ratio in the liver, both have an activated phenotype. A large proportion of liver T lymphocytes have innate-like features [132], co-expressing CD56 and/or CD161 and include NK T cells (NKT), mucosal associated invariant T cells and $\gamma\delta$ T cells, which are involved in hepatic immunity and tissue remodelling [133-135].

Innate human B cells which express high levels of CD5 are also found in the human liver and are likely to be immunoregulatory, although it is unknown whether these cells produce the

same suppressive cytokine milieu as the systemic subtype [136]. T-regulatory cell induced Breg cell formation in a mouse model of hepatic autoimmunity requires IL-21 but neither IL-10 nor TGF- β [137]. Human studies have consistently demonstrated B cell expansion in hepatitis C infected liver tissue, potentially contributing to the suppressive immune environment typically seen in chronic HCV- infected liver [136, 138-140].

In humans, NK cells account for 30 – 50% of the hepatic lymphocyte population [141], while in mice, only 5 – 10% of hepatic lymphocytes are NK cells [118, 142]. These cells have important anti-viral and anti-tumour activity and can also interact either directly or indirectly with liver APCs, KCs and DCs to regulate hepatic immune responses [143]. Several studies using murine and animal models have defined the liver's role in hematopoiesis [144-146]. Mouse studies have demonstrated that in the absence of a spleen, extramedullary hematopoiesis can occur in the liver [147]. In adult mice, the lymphopoietic capacity of adult liver cells was similar to bone marrow transplantation suggesting a key role in liver transplantation tolerance and T cell reconstitution in recipients [148]. Most human hematopoietic stem cell (HSC) studies are centred around foetal liver development although these stem cells are also present in adult liver [146]. Lymphoid progenitors (LPs) have also been detected in healthy liver tissue [33] and LPs that co-express CD 56 are more common in the liver than in the bone marrow [149]. Expansion of the HSC pool that co-expresses markers of myeloid differentiation has been described in liver metastasis [150], perhaps contributing to the increased numbers of granulocytic cells or MDSCs seen in metastatic livers.

Within the liver, a range of non-hematopoietic cell populations also possess important immune functions [118]. Liver sinusoidal endothelial cells (LSECs) are involved in hepatic leukocyte recruitment via a TLR4 activation process and appear to demonstrate both tolerogenic and proinflammatory functions on CD4⁺ and CD8⁺ interactions. Antigen-presenting capabilities have also been described in hepatocytes [151]. In response to hepatocyte antigen presentation, an effector CD8⁺ T-cell response is initiated when the initial antigen load is low in contrast to a functionally exhausted population of CD8⁺ with PD-1 expression seen in abundant hepatic antigen presentation [134, 152]. Furthermore, hepatic injury and inflammation can result in IL-10 induction by CD4⁺ T cells as a

consequence of hepatocyte activation to restore immunological homeostasis and regulate a potentially overwhelming immune response [153].

Table 1. 6 *Immunophenotypes of key hepatic immune and non-immune cell populations.*

	Surface markers
Myeloid Cells	
Kupffer cells	<i>CD45, CD68, CD11b</i>
Intrahepatic monocytes	<i>CD45, CD14⁺⁺, CD16⁻ (Classical), CD14⁺, +CD16⁺ (intermediate), CD14⁺, CD16⁺⁺ (non-classical)</i>
Dendritic cells	<i>CD45, Lin⁻, HLA-DR, CD11c, CD8α, CD11b</i>
Classic Dendritic Cells	<i>CD45, Lin⁻, HLA-DR, CD123⁻, BDCA-2⁻</i>
Plasmacytoid Dendritic Cells	<i>CD45, Lin⁻, HLA-DR, CD123⁺, BDCA-2⁺</i>
Neutrophils	<i>CD45, FPR1, CD44, CD15, CD16, CD49d⁻</i>
MDSC	<i>CD45, CD14, HLA-DR^{+/-}, CD33</i>
Myeloid progenitors	<i>CD45, CD34, HLA-DR; CD38</i>
Lymphoid Cells	
NK cells (major population in liver)	<i>CD45, CD56^{bright}, CD69</i>
NK cells (minor population in liver)	<i>CD45, CD56^{dim};CD16</i>
Liver Resident NK cells	<i>CD45, CD56, CD69, CD49 EOMES^{hi} Tbet^{lo}, TIGIT, CXCR6</i>
NKT cells	<i>CD45, CD3 CD56</i>
CD4 helper T lymphocytes	<i>CD45, CD3, CD4</i>
CD8 Cytotoxic T lymphocytes	<i>CD45, CD3, CD8</i>
Regulatory T cells	<i>CD45, CD4, CD25, CD127^{low}, Foxp3</i>
B1 B lymphocytes	<i>CD45, CD19, CD5</i>
$\gamma\delta$ T cells	<i>CD45, CD3, TCR γ/δ, CD24, CD27</i>
MAIT cells	<i>CD45, CD3, CD161</i>
Lymphoid progenitors	<i>CD45, CD34, CD56</i>
Non-immune Liver cells	
Hepatocytes	<i>Albumin, alpha-fetoprotein</i>
Stromal cells	<i>CD29, CD44, CD73, CD90, HLA-Class I</i>
Liver sinusoidal endothelial cells	<i>CD54, vWF, CD31</i>

1.5.2 Sterile injury and repair in the liver

Inflammatory activity in the liver, stimulated in the absence of foreign microbial antigens, can occur in response to a wide range of stimuli in all tissues and this is commonly referred to as “sterile inflammation” [51]. This inflammatory response can lead to the pathologically damaging production of reactive oxygen species (ROS), lipid-originated metabolites (retinoic acid and endocannabinoids), and damage-associated molecular patterns (DAMPs) which amplify inflammatory signals through Toll-like receptors (TLRs), nuclear/neuronal receptors, and the inflammasome (Figure 1.8). While maintaining an overall tolerogenic environment, the healthy liver is well equipped to restore homeostasis after disturbance.

Large populations of innate immune cells clear senescent, injured or apoptotic cells, as well as microbes and their associated products [52]. Activated neutrophils and cytotoxic cells that are no longer responding to inflammatory signals or that have already completed their immunologic function and need to be removed from the circulation migrate to the liver to die by apoptosis. This ‘detoxification’ mechanism or resolution is largely mediated by KCs, which are the first to encounter cells and molecules from the systemic circulation. High quantities of microbial DAMPs and MAMPs arriving with portal venous blood bind to pattern recognition receptors (PRRs) expressed on KCs and some hepatocytes, facilitating their phagocytosis and degradation without initiating an overwhelming immune response that usually accompanies PRRs, immediately restoring homeostasis [53, 54]. In a cirrhotic mouse model, endotoxin accumulation appeared to result from decreased KC uptake and low levels of TNF alpha release [55]. In this regard, hepatic detoxification ensures that inflammatory antigens from the gut do not travel to the rest of the body causing excessive immune activity [56]. While it is unsurprising that failed detoxification mechanisms are pathognomonic of hepatic failure predicated on the assumption of immunoregulatory disturbances, few studies have defined the inflammatory interplay in hepatic detoxification and its clinical implications.

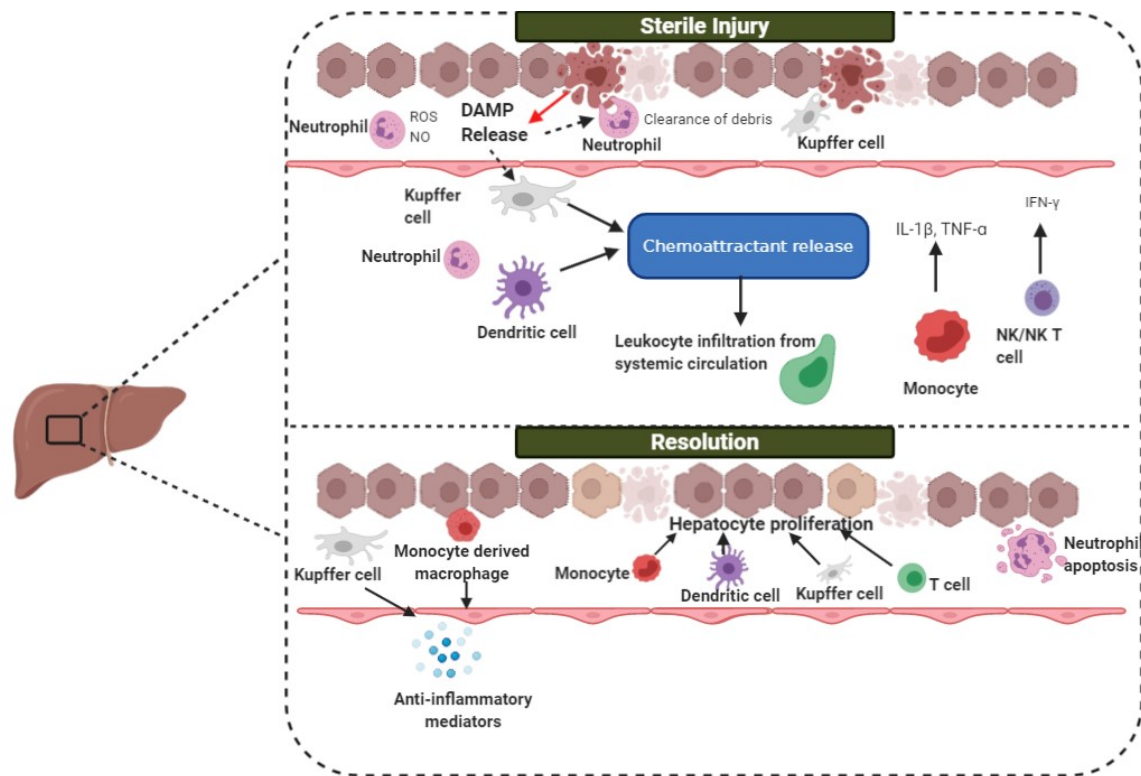


Figure 1. 8 *The acute response to sterile injury in the liver*[114]. During liver injury, hepatocyte cell death causes DAMP release resulting in Kupffer cell activation and neutrophil recruitment to the site of injury while also activating hepatic stellate cells leading to cytokine secretion. Kupffer cells and neutrophils release inflammatory mediators and begin to phagocytose necrotic debris. T cells, dendritic cells and Kupffer cells facilitate leukocyte infiltration from the systemic circulation by releasing chemoattractants such as CCL2 and IL-17. NK and NK T cells release IFN γ stimulating the release of pro-inflammatory mediators. During resolution, monocytes acquire a reparative phenotype and macrophage reprogramming occurs in a subset of macrophages to display a pro-resolution phenotype. Monocytes migrate across the liver sinusoidal endothelial cell layer, differentiating into macrophages to either replenish the Kupffer cell pool or restore homeostasis by secreting anti-inflammatory cytokines and promoting angiogenesis. Following injury, a restorative macrophage phenotype emerges promoting neutrophil apoptosis. Hepatocyte proliferation occurs to replace the lost parenchyma [154].

Some NK cells respond rapidly to injury following either direct lipid recognition or cytokine secretion by other immune cells, leading to the downstream accumulation of additional macrophages and neutrophils to the liver [57]. In murine models, type 1 NKT-deficient mice lack neutrophil and macrophage infiltration in response to injury and display hepatic resistance to ischaemia reperfusion injuries or a high fat diet suggesting that one subset of NKT-cells promotes injury [58-60]. In contrast, sulfatide reactive type 2 NKT cells demonstrate an opposing role in hepatic IRI by protecting against injury and a novel anti-inflammatory mechanism has been proposed to exploit this process and ultimately result in NKT cell recruitment in liver injury [60].

This response to acute sterile inflammation is physiological, self-limiting and results in the restoration of tissue homeostasis. However, the on-going presence of stimuli driving chronic sterile inflammation leads to pathology. These stimuli include alcohol metabolites that can induce hepatocyte cell death, excessive fat-deposition within hepatocytes resulting in lipotoxicity and oxygen depletion during ischaemia and reperfusion leading to ischaemic damage.

1.5.3 Pathogenesis of Non-alcoholic fatty liver disease

Changes to the hepatic immune environment characterise the development of non-alcoholic steatohepatitis (NASH) and murine and animal studies are defining the specific immunological events that occur throughout its clinical course (Figure 1.9). Innate immune activation and dysregulated inflammation have both been implicated in the loss of tissue homeostasis and are a subject of considerable focus. Lipotoxicity, resulting from aberrant lipid accumulation as seen in prolonged nutrient excess or obesity exceeding adipose tissue capacity, underlies the generation of several harmful signalling intermediates [155]. When storage is overwhelmed, lipid deposition occurs in ectopic sites, such as the liver, interfering with local immune regulation and resulting in a cyclical circumstance of persistent metabolic dysregulation or “metainflammation” [155, 156]. Inflammatory immune activation within the liver induces the infiltration of several other immune cell subsets and drives the progression of hepatic injury.

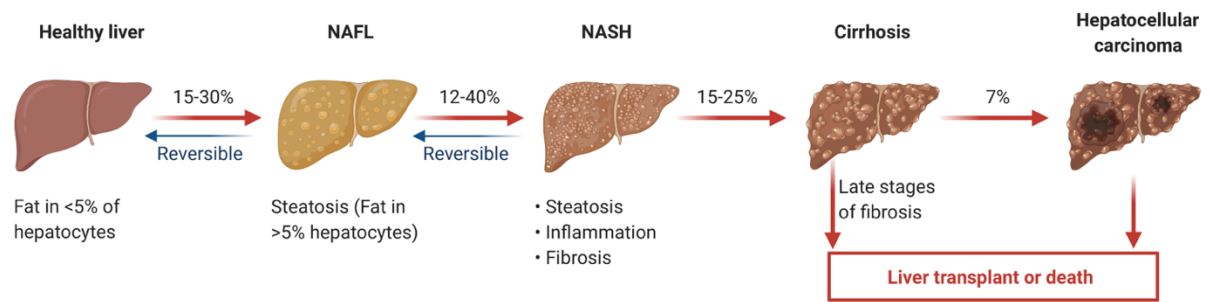


Figure 1. 9 *The spectrum of non-alcoholic fatty liver disease*. The prevalence of non-alcoholic fatty liver (NAFL) ranges from 15 – 30% of the current population. Steatosis is defined when there is an abnormal accumulation of lipids in more than 5% of hepatocytes. Non-alcoholic steatohepatitis (NASH) occurs when steatosis is combined with inflammation and cell damage. This results from mitochondrial dysfunction and oxidative stress as well as the excessive production of proinflammatory cytokines and cell death. Cirrhosis is the consequential end stage scarring from chronic inflammation which can progress to malignant hepatocellular carcinoma when the liver loses the ability to regenerate and control the physiologic cell cycle.

1.5.4 Immune consequences of Non-alcoholic fatty liver disease

KCs appear to play a pivotal role in NASH initiation and pathogenesis [157]. Concurrent activation of KCs is seen following the introduction of a high fat diet or methionine/choline deficient diets in several murine models, indicating their early involvement in the NASH complex [158, 159]. Human studies have also corroborated these findings, and KC activation and aggregation were demonstrated in NASH samples supporting their direct role in lipid processing and meta-inflammation [160]. Thus, KC activation could be linked to direct lipid uptake or lipid metabolism and the resultant lipotoxicity presenting as foreign particles [161]. In human studies, KC expansion was a key step in hepatic inflammatory initiation that, through a complex inflammatory network, results in the downstream activation of other components of the NASH complex [162]. Furthermore, there is additional evidence to suggest their pathological role in hepatic insulin resistance upon activation, further advancing discussions on the intricate interplay of immunological events and metabolic pathways in liver disease [158].

The process of circulatory monocyte differentiation into macrophages upon arrival into the liver environment is poorly understood, however, monocyte infiltration may impact some of the inflammatory responses observed in NASH. In response to hepatic injury, Ly-6C^{hi} monocytes differentiate into Ly-6C⁺ macrophages stimulating proinflammatory and profibrogenic processes [157]. In murine models, diet-induced hepatic steatosis generated Ly-6C^{hi} monocyte recruitment via CC-chemokine receptor 2 (CCR2) interactions which are largely expressed by KC and HSCs [163]. Activated monocytes recruited following hepatic injury are also guided by the expression of CC chemokine ligand 2 (CCL2) and reduced inflammatory macrophage levels were seen in CCL2 knock-off mice following an MCD diet induced NASH [164]. By contrast, an anti-inflammatory, tissue-protective Ly-6C^{low} phenotype can emerge when apoptotic hepatocytes are phagocytosed and these act to restore tissue homeostasis [165].

Moreover, KC polarization into M1 and M2 phenotypes further reveals the cyclical and overlapping landscape of the pro-inflammatory and immunoregulatory events in NASH reflecting the tolerogenic and immunogenic nature of hepatic physiology [166, 167]. For instance, M1 phenotype activation oversees the production of several proinflammatory

cytokines and chemokines such as TNF- α , IL-1 β , IL-12 and CCL2, and CCL5, advancing additional hepatocyte injury and DAMP release which further exacerbates KC activation and results in a vast influx of monocytes [124, 157]. In contrast, the role of M2 phenotypes, although largely undefined in NASH, involves inflammatory resolution and healing processes to counteract the initial recruitment of various cellular instruments [157, 167]. To date, few studies have examined M2 phenotypic changes in NASH versus non-NASH in humans, although there is evidence to suggest PPAR- δ involvement in M2 activation, which may offer a therapeutic opportunity in the clinical management of the entire NAFLD disease spectrum [168]. Additionally, mice fed with a 30-week NASH diet exhibited substantial KC loss driven by genetic changes to KC enhancers and subsequent cell death [169]. Consequently, increased expression of TREM2 and CD9 in several KC phenotypes is seen to emerge and can reflect the severity of steatosis and inflammatory injury in NASH [170, 171]. Recent single-cell analysis has further confirmed and identified the appearance of this scar-associated macrophage in murine NASH models and cirrhotic human livers [172, 173]. This TREM2⁺CD9⁺ macrophage subset induces collagen expression by HSCs and their frequency correlates with fibrosis scoring and disease severity in NASH [173, 174].

Collagen deposition resulting from hepatic stellate cell activation has been linked to the influence of profibrogenic factors through a KC activation mechanism and *in vivo* work has indicated KC depletion could attenuate fibrosis development in late disease [175]. Small animal studies and patient series have reported increased levels of activin-A in liver disease, a multifactorial cytokine belonging to the TGF β superfamily, leading to the downstream activation of KCs [176, 177]. In a mouse model, the expression of proinflammatory molecules resulting from activin-A KC activation further induces HSC-dependent fibrogenesis, seen in the later stages of NASH cirrhosis [178]. KC activation promotes HSC trans-differentiation into myofibroblastic-HSCs which leads to the production of extracellular matrix (ECM) elements, cytokine secretion and alpha smooth muscle actin (α -SMA) expression necessary for the progression of fibrosis and hepatic scarring [122].

Combined with *in situ* macrophage activation and monocyte recruitment, hepatic infiltration by additional cellular subtypes such as lymphocytes and neutrophils contributes to the underlying inflammatory disturbances accompanying NASH [156]. Following highly

orchestrated immune processes in NASH, large amounts of proinflammatory cytokines and chemokines mediate the influx of additional populations of CD4⁺/CD8⁺ T and B lymphocytes, which further enhance macrophage activation and inflammatory activity [156]. Both CD4⁺ and CD8⁺ populations are found at increased levels in blood from NASH patients and the degree of portal inflammation with CD8⁺ cells correlated with disease severity [179, 180]. In high fructose murine models, CD8⁺ deficiency was associated with steatosis resistance compared to controls [181]. Recent evidence has suggested the role of B lymphocytes in regulating T cell activation in NASH, although some of the evidence has been conflicting [156]. It is unclear whether B cell lymphopoiesis is enhanced or compromised in obesity states, however, B cell derived IL-10 has been shown to inhibit proinflammatory cytokines in metabolic syndromes [182].

1.6 Ischaemic liver injury and ischaemia reperfusion

1.6.1 Oxygen gradients within the liver

The dual blood supply of the liver consists of oxygenated blood delivered via the hepatic artery accounting for 20% of hepatic inflow with the remaining 80% delivered by the portal venous system. The healthy liver, therefore, has characteristic zones of high and low oxygen levels forming an oxygen gradient necessary for hepatic function, a phenomenon described as liver “zonation,” which is required to maintain the structural integrity of hepatocytes (Figure 1.10) [183, 184]. The concentration of oxygen decreases along the sinusoids as blood passes from Zone 1 (peri-portal) through to Zones 2 (transitional) and 3 (peri-venous). This understanding of zonation has important implications for homeostasis and various metabolic capacities can be found preferentially in one or the other zone [185]. Although the underlying regulatory pathways of oxygen zonation within the liver remain to be fully elucidated, both complementing metabolic activities and spatially separated opposing pathways in carbohydrate, amino acid and lipid metabolism have been shown to occur within distinct oxygen zones [186].

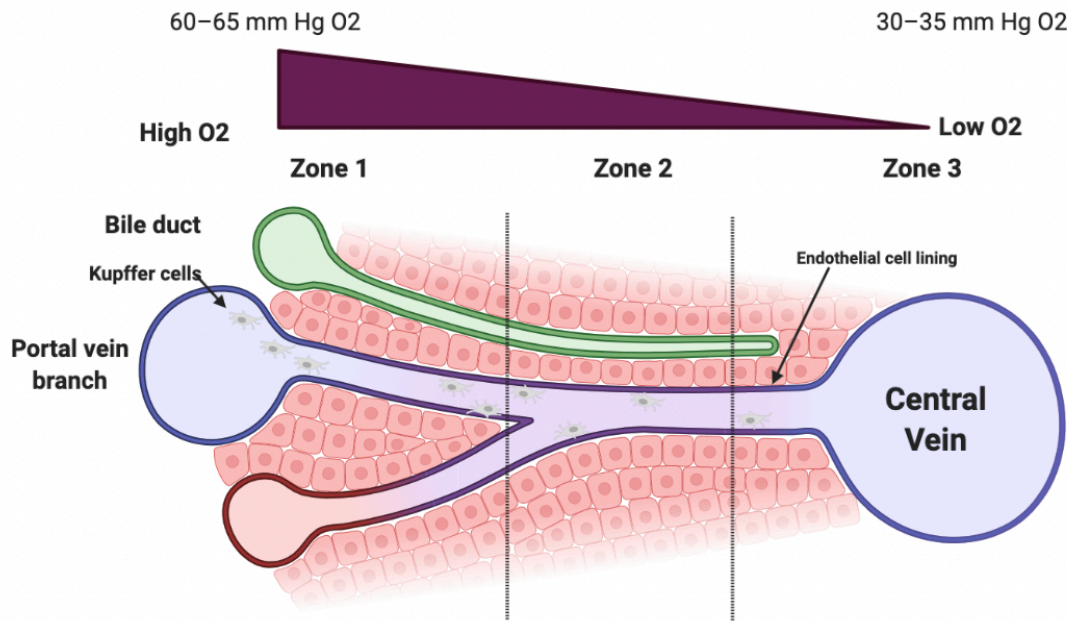


Figure 1. 10 *Oxygenation within the liver*. The oxygen gradient decreases in blood as it travels through the hepatic sinusoids (Zone 1) towards the peri-central region (Zone 3), significantly altering the local microenvironment and influencing immune cell localisation and activity. Kupffer cells are larger and more numerous in peri-portal zones compared to peri-venous zones [187].

In ischaemic injury, the absence of oxygen causes a disruption of this finely tuned gradient resulting in several functional changes at a cellular level in response to oxidative injury to counteract and control the damage. Hypoxia inducible factors (HIF) play a critical role in maintaining homeostasis. But despite the liver being exposed to variable and oftentimes low oxygen tensions, hypoxic responses are not induced in normal situations [184, 188]. During prolonged periods of severe ischaemia, as seen during transplantation and hepatic resections, HIF activation supports oxygen-independent ATP generation to support cellular processes and upregulates cell preservation mechanisms through anti-oxidant and anti-apoptotic systems that allow for cell survival. Unsurprisingly, hypoxia results in a pro-inflammatory response by increasing IFN- γ , MHC II and co-stimulatory molecule expression by hypoxic macrophages to generate T-cell driven cytokine production [189, 190]. In addition, both human and murine models have been utilised to study adaptive immune responses and T-cell recruitment in “inflammatory hypoxia” and selective FoxP3 induction and upregulation plays a key role in homeostasis by eliciting potent anti-inflammatory responses to limit overwhelming hepatic injury via Treg signalling [191, 192]. Such data

suggests the complementary role of HIF aligning with hepatic immunomodulation and tolerance, yet the extent of these mechanisms requires further clarification.

1.6.2 Immune responses during ischaemic injuries

Kupffer cells activated during ischaemia produce reactive oxygen species (ROS), TNF α , and IL-1 β resulting in subsequent leukocyte recruitment, hepatocyte death and endothelial damage [157, 193]. In addition, ROS and cytokine secretion activates CD4⁺ T cells and Natural Killer T (NKT) cells which produce IFN- γ advancing KC activation [194]. Widespread cytokine activation also upregulates the expression of T cell-relevant cell surface adhesion molecules on sinusoidal endothelial cells (SECs), and ROS production by hepatocytes triggering a multicellular immune process contributing to an overwhelming inflammatory response [195, 196]. The end result is a complex communication network of immune cells propagating ischaemic injury (Figure 1.11).

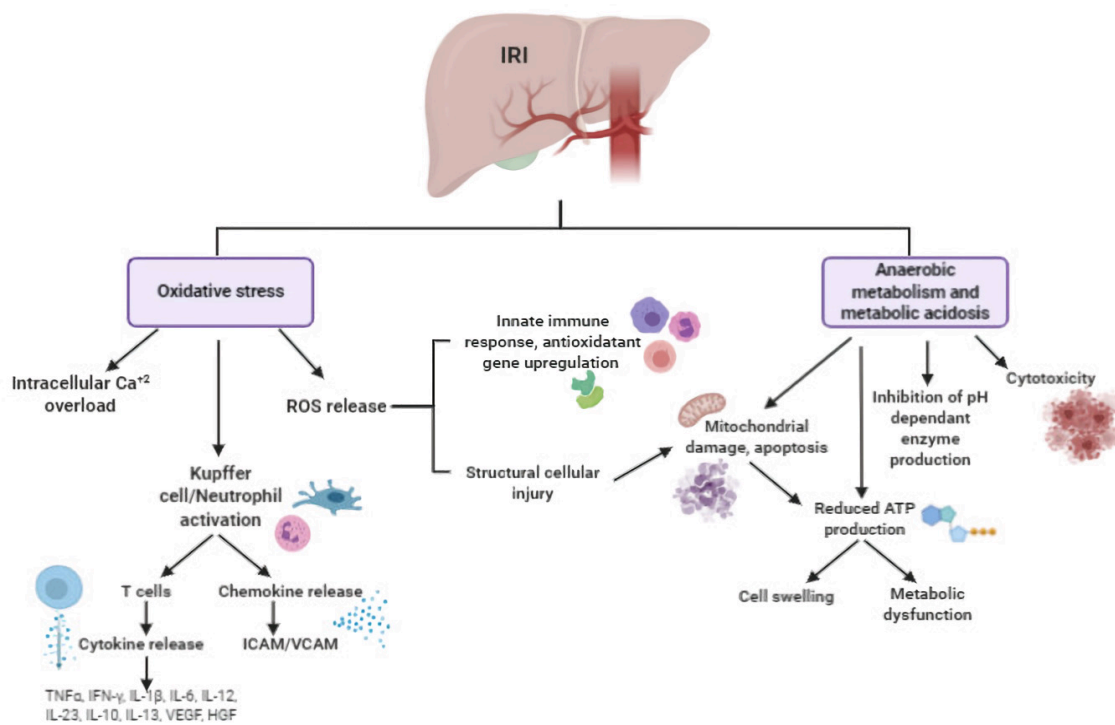


Figure 1. 11 *Physiological repair systems in ischaemia reperfusion injury*. The mechanisms of hepatic ischaemia reperfusion injury (IRI) are complex and result in a series of cellular responses influencing inflammatory, metabolic and antioxidant pathways[197]. Tissue hypoxia, oxidative stress and the resulting anaerobic cell metabolism leads to structural

cellular injury and a large amount of chemokine and cytokine release to enhance downstream immune cell accumulation. ATP: Adenosine triphosphate; IL: Interleukin; ROS: Reactive oxygen species; IRI: Ischaemia-reperfusion injury; IFN- γ : Interferon-gamma; ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule; TNF: Tumor necrosis factor; HGF: hepatocyte growth factor; VEGF: vascular endothelial growth factor.

Hepatic immunity during ischaemia is mainly focussed on immune restoration and regeneration without generating detrimental immune responses in normal scenarios. As mentioned previously, liver zonation and the maintenance of a fine oxygen gradient is required to support the structural integrity of healthy hepatocytes [183]. As such, the pathological consequences of ischaemia are particularly apparent during ischaemia reperfusion injuries (IRI), a widely accepted example of sterile inflammation, with the rapid restoration of oxygen to ischaemic tissue triggering apoptosis and oncotic necrosis [198, 199]. Ischaemia can be categorised into warm or cold, depending on the temperature at which it occurs (Figure 1.12). While warm ischaemia is known to cause major disruptions to molecular signalling and cellular pathways, cold ischaemia has been considered protective due to reduced ATP demands for regular molecular processes. For this reason, cold ischaemia is being utilized in liver surgery, especially transplantation, to minimize the injuries encountered during warm ischaemia. In both instances, the physiological repercussions are proportional to the extent of ischaemia and duration of oxygen blockade.

1.6.2 Immune responses during reperfusion

Reperfusion is divided into two phases. The first early phase can last up to 2 hours after reperfusion and sees the activation of KCS, SECs and the inflammatory effects of ROS [200]. The second late phase lasts between 6 to 48 hours after reperfusion and results in cellular degradation by neutrophil infiltration, and CD4⁺ lymphocytes which release proteases and cytotoxic enzymes [201, 202].

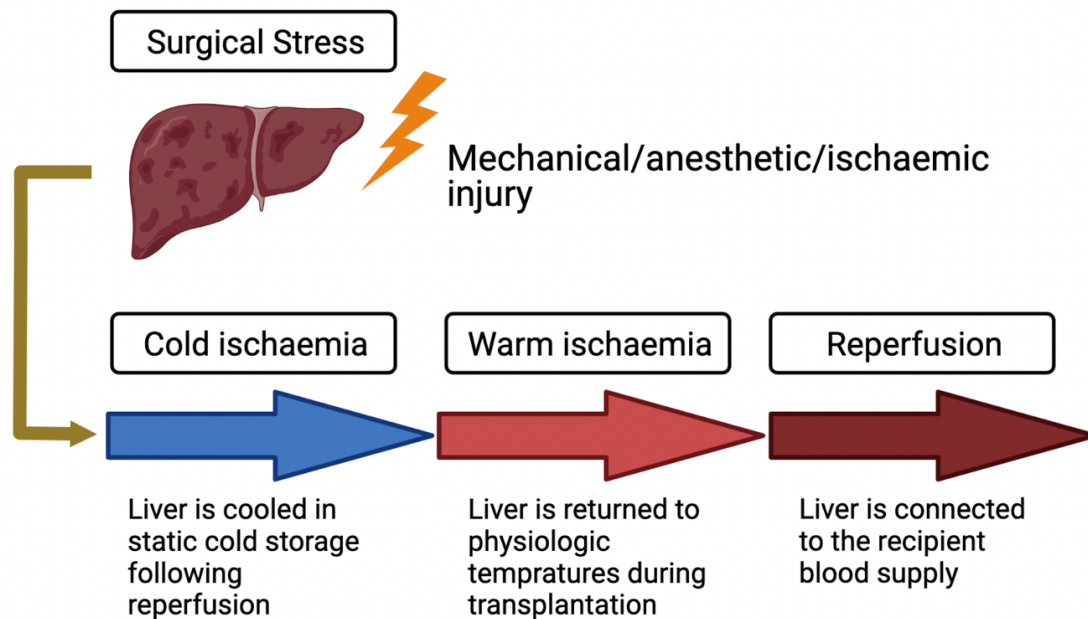


Figure 1. 12 *Phases of ischaemia during liver transplantation*. Major surgery is associated with a significant systemic response and the liver attempts to combat the inflammatory imbalance and surgical stress in order to maintain homeostasis. In liver transplantation, the liver is initially cooled, traditionally in static cold storage, following procurement to slow metabolic processes and dampen the immune response. This cold phase lasts for several hours and ends the moment the graft is taken out of cold storage. Although the liver is cooled down, cellular processes are reliant on an anaerobic environment and cells can only continue in this phase for a limited amount of time before cell death occurs. During the subsequent rewarming phases, the graft is highly susceptible to injury. The first two “ischaemic” phases cause initial functional changes that are further exacerbated by reperfusion. Although the liver is warmed up to physiologic temperatures, anaerobic conditions still persist in the “warm ischaemia phase”. Paradoxically, when the liver is connected to the recipient blood supply, restoring aerobic conditions, the initial influx of oxygenated blood releases a burst of ROS causing oxidative damage.

Ischaemic injury and IRI, although oftentimes used synonymously, are distinct entities. During ischaemia, hepatocytes are disrupted immediately and metabolic processes shift from oxidative phosphorylation to glycolysis [199]. Subsequent ATP depletion and calcium accumulation leads to necrosis in the absence of ROS, a distinguishing feature from the pathological processes of IRI [199, 203]. In routine hepatic surgery, ischaemic time to reperfusion and tissue re-oxygenation, whereby hepatocyte leakage results in DAMP release and subsequent KC activation which is the main driving factor in ROS production, is limited in non-emergent cases [199, 204]. In addition, activation of the complement system and its components, of which several are potent neutrophil activators, contribute to the widespread inflammatory cascade during reperfusion [205, 206].

Liver parenchymal condition strongly determines the extent of ischaemic injury, and several animal models have established reduced hepatic regeneration and more extensive IRI on the background of chronic liver disease. It has been demonstrated in murine models that steatotic and cirrhotic livers exhibited more devastating inflammation and hepatic injury following IRI with a slow ability to recover normal cellular functioning and healing [193, 207, 208]. Molecular aberrations in older livers are also likely to weaken immunotolerogenic responses to ischaemic injury. Older livers demonstrate reduced tolerance to IRI and a consistent inability to regenerate, partly explaining why younger donors are considered optimal in the transplant setting [209, 210]. In a mouse model, mature adult mice displayed significantly less neutrophil accumulation, suppression of the NF- κ B inflammatory transcription factor and almost complete absence of the macrophage inflammatory protein-2 necessary for neutrophil recruitment [211]. Tissue remodelling roles for neutrophils have also been described in IRI. In a model of sterile liver injury, neutrophils were shown to dismantle injured vessels and create channels for new vascular growth [212], while in a chronic liver injury model, neutrophils promoted fibrolysis and suppressed the development of fibrotic lesions [213].

A significant proportion of harvested donor livers are rejected because of fat deposition and probable damage caused by fat induced sterile inflammation. Fat-related damage is likely to significantly enhance IRI during transplantation and result in subsequent graft loss [214]. A

major aim in liver transplant research is to provide some sort of protection in order to minimize IRI, particularly in marginal livers, to withstand surgery [215].

1.7 Anti-oxidants, reactive oxygen species and homeostasis

1.7.1 Oxidative stress

As part of its role in first-pass effect, the liver is a major site in pre-systemic metabolism and therefore becomes exposed to high quantities of various compounds that produce free radicals and oxidative damage by reactive oxygen species (ROS) [216]. During hepatic metabolism of xenobiotics and endogenous molecules, several free radicals are formed [217]. ROS are produced during normal intracellular metabolism from several endogenous (mitochondria, xanthine oxidase, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation) and exogenous sources and play a critical role in modulating gene expression, molecular pathway messaging and cellular defense [218]. Oxidative stress refers to the imbalance between the formation of free radicals (FR) and the body's anti-oxidant defenses (Figure 1.13) [219, 220]. The accumulation of pro-oxidants results in eventual cellular lipid peroxidation, oxidative DNA injury and protein damage [220].

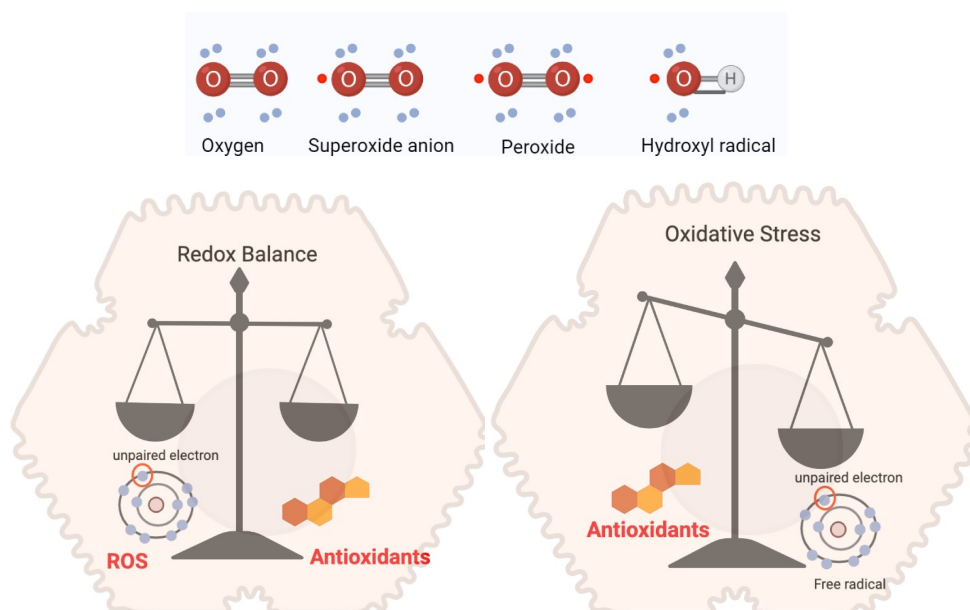


Figure 1. 13 *Redox balance*. Reactive oxygen species are by-products of normal cell metabolism of oxygen and carry physiological roles at moderate and low concentrations. ROS include radical species such as superoxide ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), along with nonradical species such as hydrogen peroxide (H_2O_2). Healthy cells maintain an intricate balance of redox homeostasis whereby ROS levels are finely tuned by an antioxidant defense system. Oxidative stress is the result of the imbalance between oxidants and antioxidants causing further disruptions in redox signaling and molecular damage.

The term “Redox”, initially formulated in 1985, incorporates two important processes ‘reduction’ and ‘oxidation’[221]. Reduction involves a decrease in oxidation whereby a gain in electrons occurs, whereas in oxidation, electron loss occurs [222]. Sources of ROS can be both endogenous and exogenous. Endogenous sources in mammalian cells include dinucleotide phosphate (NADPH) and oxidases and intracellular enzymes which can generate ROS during enzymatic function such as the cytochrome p450 system, cyclooxygenases and xanthine oxidase. Exogenous factors that can contribute to ROS production are ultraviolet light, pollutants, tobacco and radiation [118].

ROS play a dual role in cell function and can be either beneficial or harmful depending on concentration levels and source induction. At relatively low or moderate concentrations, ROS play a role in immune defense by inhibiting bacterial metabolism and growth [223], form a critical component of the mitogenic signaling pathway [224] as well as regulating several diverse physiologic parameters and enzymatic activity in the body [225]. Evidence suggests that “good” ROS are by-products of the mitochondrial respiratory chain and most of the physiological effects appear to be mediated by ROS derivatives of superoxide which form during the first few minutes of reperfusion [226]. In contrast, high concentrations of ROS can be harmful and results in biological damage from unchecked increased levels [227]. Evidence suggests that during ischaemia, the respiratory chain, redox active enzymes and NADH and CoenzymeQ (CoQ) are reduced [228]. Oxidant injury can occur at a transcriptional level implicating signal transduction levels and mRNA stability, dysregulating physiological stress proteins and DNA repair proteins and disrupting mitochondrial electron transport proteins [229]. High ROS levels can cause oxidative DNA damage and mutagenesis [230] giving rise to several cancerous and non-cancerous conditions[231]. In particular, excessive and unbalanced ROS generation has been implicated in the development and progression of pulmonary, cardiovascular, metabolic and neurodegenerative disorders [232] (Figure 1.14). To counteract oxidative stress and maintain the delicate antioxidant/prooxidant balance, intricate antioxidant defense systems consisting of endogenous antioxidant enzymes and non-enzymatic antioxidants exist in healthy humans [229].

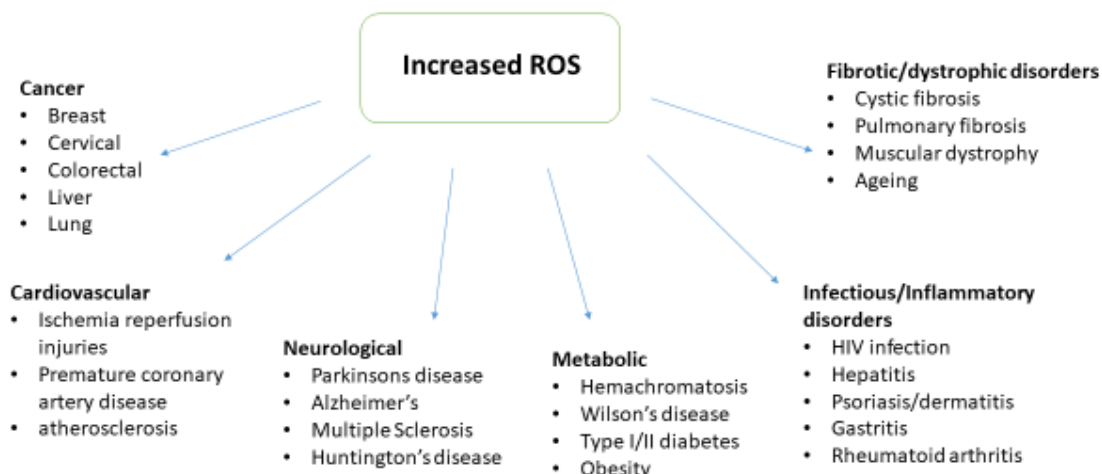


Figure 1. 14 Schematic representation of pathological conditions which occur from excessive and unchecked ROS production.

1.7.2 Antioxidant systems

In the liver, parenchymal cells contain most of the hepatic anti-oxidant enzymes, however, free radicals specifically affect hepatocytes, endothelial cells, Kupffer cells and hepatic stellate cells which are more sensitive to ROS by activating myriad inflammatory pathways, ischaemia, cell death and combining with proteins, fatty acids and DNA [218, 233, 234].

Oxidative stress can specifically trigger hepatic injury by inducing excessive fatty acid oxidation, lipid peroxidation, inflammation, apoptosis and increased collagen synthesis [235]. Reducing oxidative stress is dependent on a functioning anti-oxidant system within the cell. As ROS play a role in multiple signalling pathways and mechanisms, they are naturally influenced by several genes and proteins, not only in oxidative injury, but also modulating systems that control normal biological functions and cellular homeostasis.

Table 1. 7 Agents involved in ROS metabolism and anti-oxidant homeostasis. Adapted from Yang and colleagues [236].

Regulation	Proteins/genes	Effects
ROS inhibitors	NRF2	Limits ROS production
	NRROS	Reduces tissue damage
	HIF- 2 α	ROS homeostasis
	PGC-1 α	Co activator and upregulates mitochondrial antioxidant genes
	ERK	Reduces cellular injury by ROS
	PKM2	Reduces oxidative damage on cancer cells
	Ucp2	Limits ROS production in inflammatory cells
	Vitamin C/E, FHC	Suppress ROS accumulation
ROS promoters	TLR1, 2, 4	Increase ROS generation in macrophage
	NOX2	Increase mitochondrial ROS production
	p66 ^{SHC}	Increase mitochondrial ROS as apoptosis signal
	TNF	Enhance macrophage killing and necroptosis
	MMP-3	DNA damage and genomic instability
	EST-1	Increase ROS generation
	UPBEAT1	Change cells from proliferation into differentiation

Several agents are involved in ROS metabolism by either inhibiting the formation or further propagation of free radicals. The negative regulator of ROS (NRROS), is a homeostatic example of effective antioxidant defences through its role in limiting the production of ROS in response to invading pathogens and thus limiting collateral tissue injury [237]. In addition PGC-1 α has been described as a transcriptional co-activator playing a critical role in the β -oxidation of fatty acids and specifically, mitochondrial biogenesis [238]. Notably, erythroid 2-related factor 2 (NRF2) is a major regulator of cellular redox balance involved in the transcription of over 500 genes in the human genome, two dozen of which coordinate an

extensive antioxidant defence pathway, while also contributing to a broad spectrum of cellular functions including the cell cycle, immunity, protein degradation, mitochondrial homeostasis and metabolism [239].

1.7.3 The Nuclear Factor (erythroid-derived 2)-like 2 (NRF2) complex

NRF2 is a transcription factor regulating redox homeostasis as well as anti-inflammatory and cytoprotective gene expression [240]. This factor is a member of the cap n collar subfamily of basic region leucine zipper (bZip) transcription factors [241]. First reported in 1994 by Mo et al in an attempt to study the beta-globin gene locus and its regulation in erythropoiesis, the authors found NRF2 to be expressed ubiquitously in all tissues and functioning as a master regulator of the cellular antioxidant cell defence system [242].

There is emerging evidence linking IRI with the NRF2 pathway and its multimodal ability to regulate potent gene expression through an anti-oxidant response element (ARE) signalling axis [243]. In normal cellular conditions, NRF2 is kept in the cytoplasm by an inhibitory actin-bound cytosolic protein known as Kelch-like ECH-associated protein 1 (Keap 1) linking it to ubiquitin ligase complex consisting of Cullin 3 (CUL3), and ring-box 1 (RBX1)[244]. Increased ROS production liberates NRF2 from the inhibitory complex allowing it to escape proteolysis and in turn translocate into the nucleus. NRF2 then heterodimerizes with small Maf (sMAF) proteins which causes the gene induction of antioxidant response element-dependant genes (ARE) inducing a diverse array of cytoprotective axes [245-247]. Interestingly, NRF2 appears to be activated ubiquitously in the liver within hepatocytes, Kupffer cell and NK cells in response to oxidative injury to rescue cellular function and restore metabolism [248-250]. This represents a natural cellular response to oxidative stress by upregulating endogenous anti-oxidant genes to combat the ischaemic insult and a logical therapeutic target (Figure 1.15).

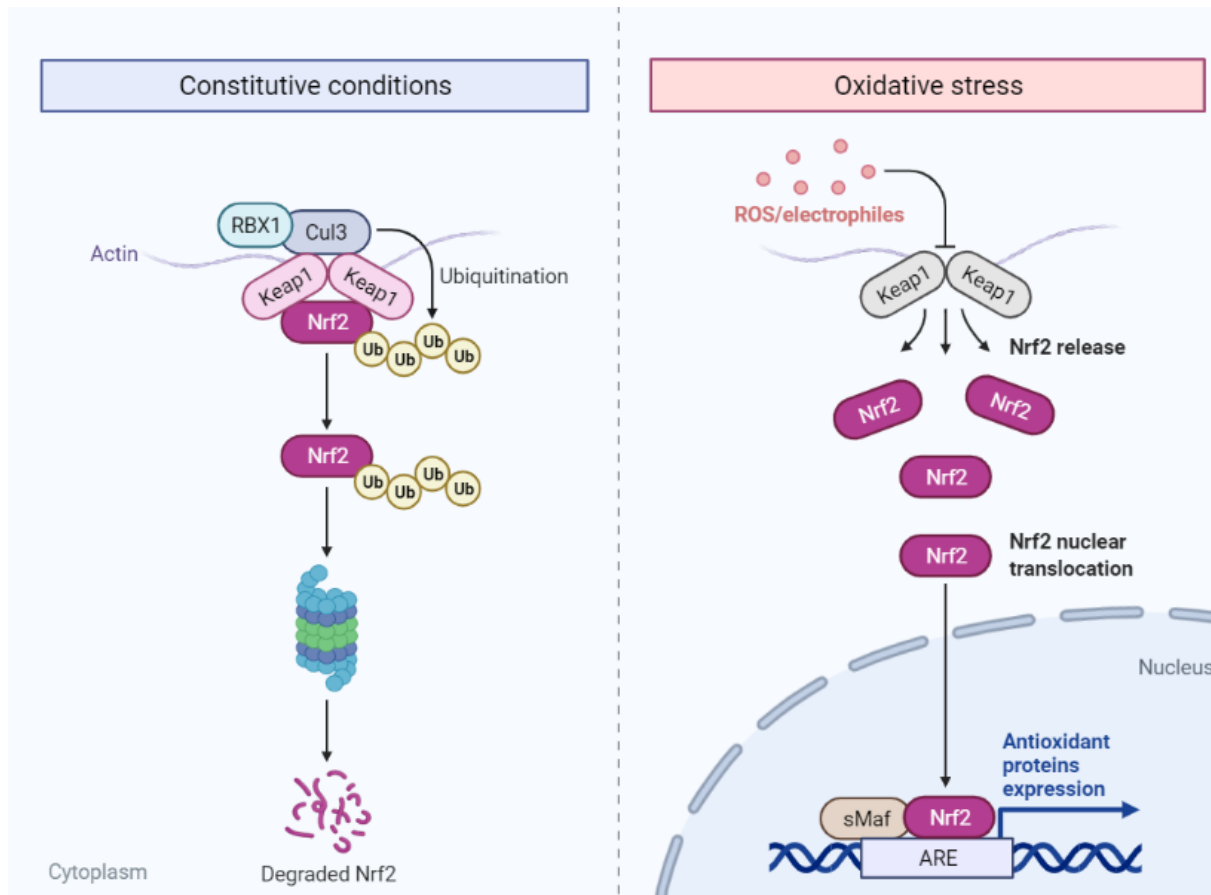


Figure 1. 15 A schematic diagram of the NRF2 pathway. In unstressed conditions, NRF2 is maintained in an inhibitory state by a ubiquitin protein ligase complex of KEAP1/CUL3/RBX1 which causes NRF2 degradation and steady –state levels. Cellular stress in high ROS conditions causes electrophilic modification of Keap 1 and the formation of disulphide bonds between its cysteine residues which causes NRF2 to escape from the inhibitory complex. NRF2 then becomes stabilised and translocates to the nucleus to form a dimer with sMAF and the induction of several antioxidant and cytoprotective genes.

Evidence suggests an autoregulatory loop exists in NRF2 signaling which controls the switching off of NRF2 (Figure 1.16). When NRF2 is activated following nuclear translocation, studies have confirmed that induction of Keap1 occurs through NRF2 binding to an ARE in the reverse strand of the Keap1 promoter. Hence, NRF2 can regulate Keap1 transcription and Keap1 can regulate NRF2 activity by degrading it [251, 252]. Interestingly, NRF2 also induces promoter activity of Cul3-RBX1 genes by binding to an ARE in both the forward and reverse strands of the proximal promoters of the Cul3-RBX1 genes which consequently allows the proteins to accelerate ubiquitination and degradation of NRF2 [244].

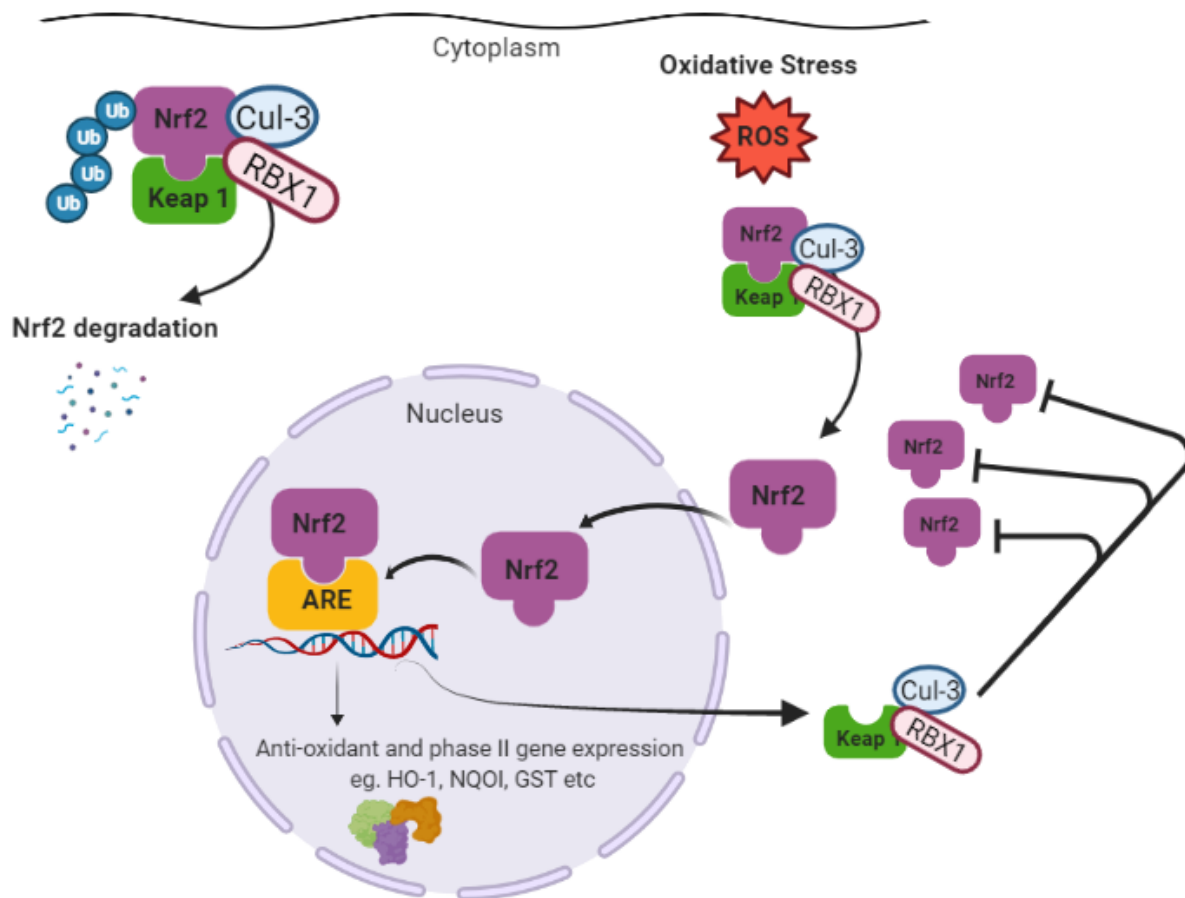


Figure 1. 16 *Proposed autofeedback loop between NRF2, Keap1 and Cul3-RBX1 inhibitory complexes.* NRF2 regulates inhibitory genes at the level of transcription. The subsequent formation Keap1 complexes NRF2 at the level of its activity [244].

While NRF2 has been considered a master regulator of intracellular redox homeostasis, evidence suggests it plays a bigger role in promoting cell growth and cell survival in environmental disruptions. Several components of the proteasomal pathway are regulated by NRF2. A specific class of proteins such as the 26S proteasomes prevent the abnormal accumulation of damaged or misfolded genes which can impact cellular function and cause apoptosis [253]. The pathological inability of proteins to degrade appropriately can give rise to several neurodegenerative and cardiac diseases [254, 255]. The expression of these proteasomal subunits by NRF2-dependant transcription was shown in mice livers following treatment with dithiolethiones [256]. Furthermore, crosstalk between the NRF2-Keap1 axis and the endoplasmic reticulum (ER) signalling network has been studied and NRF2 is considered a P-ERK substrate regulating the activity of the ERK pathway [257].

Molecular evidence suggests that NRF2 signalling also plays a role in regulating specific anti-inflammatory processes. Heme-oxygenase (HO-1) is a well-known antioxidant which is part of the ARE-dependant phase II detoxifying enzymes regulated by NRF2 [258]. In a rat orthotopic liver transplantation model, HO-1 induction prevented CD95/FasL-mediated apoptosis and resulted in a depressed expression of helper T cell type 1 cytokines (interleukin 2 and interferon γ) [259]. Similar reports have identified HO-1 as a powerful downstream effector of IL-13 which downregulates the production of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, MIP-1 α and MIP-2) [260, 261]. Furthermore, activation of the NRF2-HO-1 axis has been shown to lead toward the promotion of an anti-inflammatory M2 phenotype and inhibition of an M1 phenotype during hepatic IRI [262]. Consequently, specific NRF2 activation has been shown to reduce macrophage/neutrophil trafficking, pro-inflammatory cytokine programs and hepatocellular necrosis while also increasing anti-apoptotic functions in IR-stressed livers [263].

Previous work by Zaman and colleagues from St Vincent's University Hospital Dublin investigated whether NRF2 expression in donor livers could influence LT outcomes [247]. Data from 14 donor livers showed that livers from older donors had lower levels of NRF2 which appeared to expose these organs to a greater degree of IRI during transplant. Furthermore, livers from donors with greater NRF2 mRNA had lower Suzuki scores and better liver function after transplant.

Upregulation of the NRF2 axis has mediated IRI protection in a number of non-hepatic clinical settings, including cerebral, retinal, intestinal and cardiac tissue [264-267]. Coordinated NRF2 upregulation has also shown some benefits in treating diabetic nephropathy and naturally occurring NRF2 inducers have been reported to halt acute and chronic inflammatory liver states [268, 269]. However, the therapeutic potential of NRF2 is still unclear in liver transplantation. Additionally, the clinical relevance of the NRF2 axis on extended criteria livers also remains unexplored.

1.8 Hypothesis and objectives of the project

1.8.1 Hypothesis

We hypothesize that the NRF2 axis influences allograft quality in liver transplantation and may provide a rational biomarker for allograft selection and therapeutic targeting. The overall objective of this thesis is to assess the relationship of hepatic NRF2 expression on the functionality of discarded or marginal liver allografts.

1.8.2 Specific aims

The specific aims of this thesis are to:

1. Determine the functional expression of NRF2 in discarded human liver samples and its impact on hepatic function and histopathology
2. Investigate NRF2 changes during normothermic machine perfusion
3. Assess the relationship between NRF2 and hepatic steatosis
4. Review current strategies using NRF2 analogues in the setting of liver transplantation in order to identify a suitable agonist
5. Examine the effects of using the agonist pharmacologic NRF2 upregulation in steatotic samples in a murine ex vivo machine perfusion model

Chapter 2

Materials and Methods

2.1. PART A: Human experiments

2.1.1 Human liver samples

Ethical approval was acquired from the Washington University School of Medicine (WUSM) Institutional Review board. Samples were obtained from livers procured and declined for transplantation by the liver transplant team at Barnes Jewish Hospital between 2017 and 2020. In all cases, written informed consent was obtained from each donor family to consent to the use of livers for research purposes. Livers were procured using a standard surgical technique accessing the abdomen through a midline laparotomy[16]. After cross clamping the supraceliac aorta, the abdomen is packed with ice and organs are flushed with Histidine-tryptophan-ketoglutarate (HTK) solution through the infrarenal aorta and splenic vein. In total, samples from 40 discarded human liver allografts were collected and biobanked at -80 degrees. Patient details were extracted from the United Network for Organ Sharing (UNOS) database including donor demographics, cause of death, pre-operative biochemical serum analyses and cause of organ discard. Donor cause of death was defined by adopting the same UNOS and Organ Procurement and Transplantation Network (OPTN) nomenclature for categorical analysis.

2.1.2 Normothermic Machine Perfusion System

Our human NMP apparatus is a closed recirculating system, as previously described (Figure 2.1), consisting of centrifugal pump (CBBP50 Carmeda pediatric Bio-Pump; Medtronic Inc., Minneapolis, MN), 1 membrane oxygenator/heat exchanger (CB3381 Carmeda pediatric Minimax Plus; Medtronic Inc.), 1 dialysis machine (Fresenius 2008K, Fresenius Inc, Concord, CA), 1 trioptic measurement cell (Medtronic Inc.) for real-time monitoring of oxygen saturation and hematocrit, 2 flow probes (Pediatric Bio-Probe; Medtronic Inc.), 2 pressure monitoring probes, and an organ chamber [270]. All circuits were connected in parallel. Perfusate temperature was maintained at 37 °C by the dialyzer and heparin was infused at 500 U/h.

Before connecting the liver to the machine, the circuit was primed with 1.5L of normal saline and 10,000U of heparin. Type matched blood to the liver donor was then added to the circuit (2 – 3 units) and the system is run for approximately 30 minutes to allow the

dialyzer to equilibrate the perfusate electrolytes and pH and for the circuit to warm up. This blood was obtained from the Barnes Jewish Hospital blood bank and was either expired or nearly expired. Previous consent was attained from donors to use their blood for research purposes in the event that it would expired. The perfusate was supplemented with the following: 1000 U/hour of heparin, 7 mL/hour of total parenteral nutrition (TPN; Clinimix E4.25/5, Baxter Inc., Deerfield, IL), 20 IU/hour of regular insulin, 10 µg/hour of prostacyclin (GlaxoSmithKline Inc., London, UK), and 7 mL/hour of 1% taurocholic acid (Sigma Aldrich, MO)[270]. The liver is then flushed with 2 liters of saline through the portal vein and 1 liter for the hepatic vein before connecting to the NMP system. Hepatic artery flow rates were maintained at 0.45 ± 0.15 L/minute and portal vein pressures were kept at 1.4 ± 0.2 L/minute to ensure constant arterial pressures of 85 ± 10 mm Hg and portal pressures of 9 ± 1 mm Hg[270]. The temperature was constantly monitored and regulated using an integrated thermometer on the thermoelectric water pump (Polystat [Cole-Parmer Inc.], Vernon Hills, IL), the dialysis machine, and the integrated thermometer port on the oxygenator. Eight livers were placed on the NMP circuit and maintained at 37 °C for 6-hours following a brief storage period at 4°C after procurement.

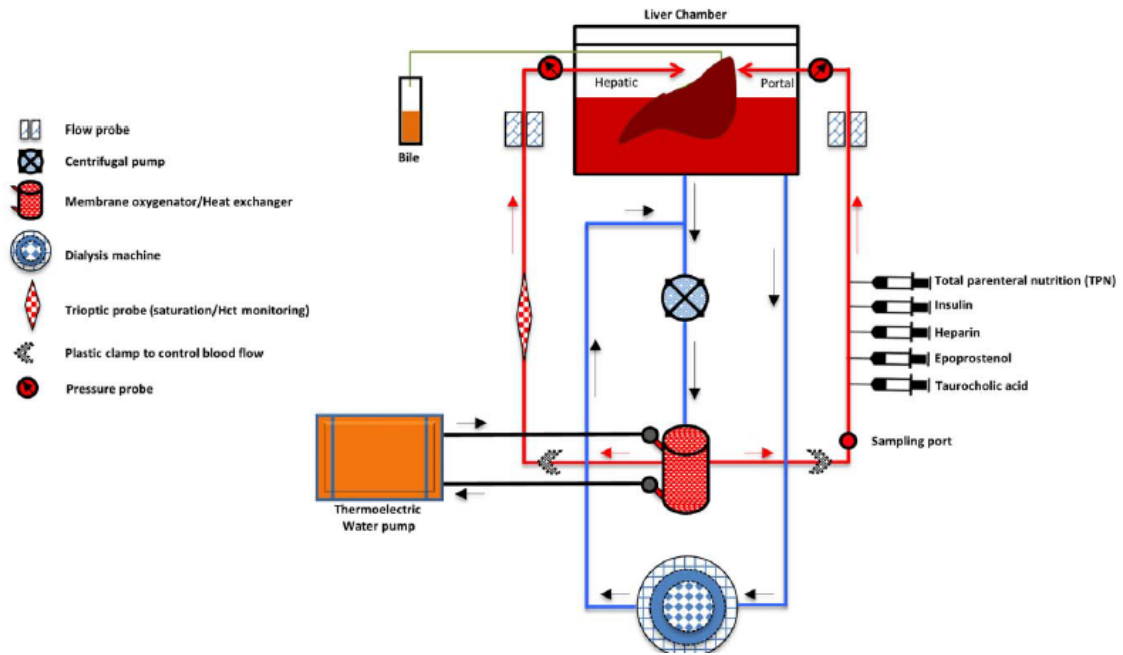


Figure 2. 1 *Normothermic Machine Perfusion Circuit for liver allografts*[271]. Livers were procured, briefly stored at 4°C for transport and connected to the NMP (dual cannulation) circuit for 6 hours of perfusion. Liver biopsy samples were taken prior to and just after NMP for staining purposes. The NMP circuit consists of 1 centrifugal pump and 1 dialyzer. The dialysate was set to K = 3 mmol/L, Ca = 3 mmol/L, and perfusate flow was set to 300 mL/minute. Blood samples were taken at hourly intervals [271].

2.2 PART B: Exploring current strategies of NRF2 targeting in liver transplantation

2.2.1 Literature search

A comprehensive review of the literature was conducted, in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, identifying all published studies utilizing an NRF2 amplifying medication in liver ischaemia reperfusion injury. Due to the paucity of data on human and murine studies, published reports on all studies were included. An electronic search was performed on five major databases (Embase, Medline, PubMed, PubMed Central and Cochrane Library). The following Boolean search terms were employed: NRF2 OR nuclear factor-erythroid 2-related factor 2 AND ischaemia reperfusion OR liver transplantation. The inclusion criteria were high quality randomized or non-randomized comparative analyses in which an NRF2 agonist was specifically used prior to or following hepatic ischaemia reperfusion. Exclusion criteria were studies which included the effects of machine perfusion, non-hepatic solid organ transplantation and review articles. In addition to the primary search, the bibliographies of selected articles were manually reviewed to identify other studies for inclusion and an additional review of 'related citations' in PubMed was also performed.

2.2.2 Study selection and data extraction

Titles and abstracts of identified studies were screened for relevance to the proposed research question, the therapeutic impact on NRF2 amplification in liver transplantation. When two publications were believed to involve overlapping populations, the most recent and comprehensive cohort was included. All search results were combined in a reference manager database (EndNote X9) and duplicates were removed. For all studies, data pertaining to author institution, country, study period, methodology, subject number (n), medication used and biochemical analyses were extracted. Study methodology was assessed using the MINORS and Jadad scales, as appropriate [13, 14]. Where animal studies were included, risk of bias was assessed using the Systematic Review Center for Laboratory animal Experimentation (SYRCLE) tool as described by Hooijmans and colleagues [15].

2.3 PART C: Animal studies

2.3.1 Rat liver procurement

Animal experiments were approved by the Washington University Animal Studies Committee and Department of Comparative Medicine. Eight male Sprague Daley rats (Charles River Laboratories, USA) were obtained at 5 weeks and fed a 60% high fat diet for a total of 8 weeks (Research Diets Inc.). Animals were housed under controlled environmental conditions with a 12:12 hour light/dark cycle and free access to water. Anesthesia was induced with 4% isoflurane (Sigma, St Louis, MO, USA) and animals were later maintained on 2% with continuous oxygen flow. The abdominal cavity was accessed through a midline incision and the liver was recovered using a modified version of the cuff technique of standard dissection and hepatic isolation [17, 18]. The abdominal aorta was cannulated and the animal was heparinized with 300 U/kg. Blood was then collected in blood collection bags (Jorgensen Laboratories, Inc., Loveland, CO). The supraceliac and infrarenal aorta were ligated, the portal vein was cannulated and cardiac arrest was induced using KCL (75-100 mg/kg) as previously reported [271]. The livers were flushed with 3 liters of HTK solution that was stored at 4 °C. The livers were then benched and cannulated on the back table prior to placement onto the rodent NMP circuit.

2.3.2 Rodent Normothermic Machine Perfusion System

We used a modified NMP system based on the previously reported Harvard Apparatus (Holliston, MA)[19]. Whole rat blood from lean rat blood donors (70mL – 110ml per rat) was collected to act as perfusate for the machine system. The circuit was first primed with 700ml of normal saline and 10,000 U of heparin. We have described our rodent NMP system in previous studies (Figure 2.2) [270, 271]. This was a single recirculating circuit consisting of 1 centrifugal pump (CBBP50 Carmeda pediatric Bio-Pump; Medtronic Inc., Minneapolis, MN), a liver chamber that served as a blood reservoir, a membrane oxygenator/heat exchanger (CB3381 Carmeda Pediatric Minimax Plus; Medtronic Inc., Dublin, Ireland), 2 flow probes (Pediatric Bio-Probe; Medtronic Inc.), and a dialyzer (Fresenius 2008K)[271]. The perfusate temperature was controlled using a thermoelectric water pump (Polystat; Cole-Parmer, Vernon Hills, IL) connected to the integrated heat exchanger of the oxygenator, and heparin was infused at 500 U/hour. The total perfusate volume for the machine was 20mL per liver

perfused on the apparatus. Livers were placed on the NMP circuit and NRF2 agonist (Oltipraz, ab141957) or DMSO control vehicle were delivered at the initial 1-hour time point when the system temperature was stabilized and perfusion was maintained at 37°C for an additional 3 hours. Perfusate samples were collected at baseline (0hr) and 4-hours of NMP for blood gas and biochemical testing. Rat liver tissue was collected at both time points for histological analysis.

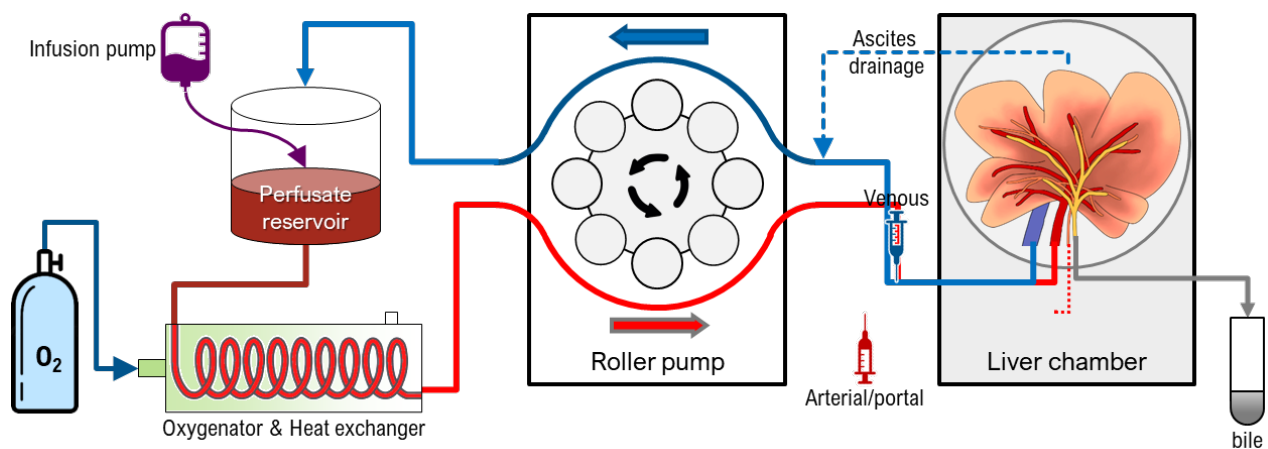


Figure 2. *Ex vivo machine perfusion for the rodent model.* Rat livers were procured and immediately connected to the NMP system for a total of 4 hours with an NRF2 agonist or vehicle control added into the perfusate at the 1 hour timepoint.

2.4 PART D: Liver assessment and benchwork

2.4.1 Western blot

Western blot analysis was performed as previously described [20]. Approximately 100mg of frozen tissue was homogenized using a homogenizer 3 times for 10 seconds each. Protein lysis was performed at 4°C to prevent proteolytic degradation of the total proteins. Ten volumes of Radioimmunoprecipitation assay buffer (RIPA) (10x CST #9806) lysis buffer containing a cocktail of protease (Thermo Scientific #88665) and phosphatase (Thermo Scientific #A32957) inhibitors were added to each homogenized tissue sample. Following 5 minutes of centrifugation at 10,000 rpm, the supernatant was recovered and protein content was determined using an Invitrogen kit ([Q33211](#), Invitrogen). Prepared homogenates were aliquoted and stored at -80°C until required for experimental analysis. For each sample, 50 µg/well of total protein, lysate was loaded onto a 4–12% Nu-PAGE Bis-Tris (Invitrogen) gel and size fractionated using 2 hours of electrophoresis at 80V. Proteins were then transferred from the gels to Poly(vinylidene fluoride) (PVDF) membranes (1620177, BIO-RAD) in a semidry apparatus at 30 V for 1.5 hours, membranes were blocked (5% milk) and subsequently probed for NRF2 expression initially using an anti-NRF2 rabbit antibody (ab137550) followed by secondary antibody incubation (CST#7074) and for additional relevant antibodies (Table 2.1). Protein bands were developed using an ECL Kit (CST#6883S) and images were generated with iBright CL1000 imaging. Protein band intensity was measured and quantified using GIMP software (GIMP 2.8, CNE, Free Software Foundation, Boston, USA). Protein stain intensity was interpreted as a measure of functional protein expression. Attempts were made to prepare separate nuclear and cytoplasmic tissue extracts to determine protein expression. These, however, were unsuccessful and were felt to be attributed to the quality of the frozen tissue samples and the degree of parenchymal injury.

Table 2. 1 *List of antibodies used for Western Blotting*

Antibody	Supplier	Isotype	Type	Block	Primary Antibody
Anti- β -Actin	Cell Signaling	Mouse	Monoclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-GAPDH	Cell Signaling	Rabbit	Monoclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-CCP-3	Cell Signaling	Rabbit	Polyconal	5% non fat dry milk	1:1000 o/n 4°C
Anti-tAMPK	Cell Signaling	Rabbit	Polyclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-pAMPK	Cell Signaling	Rabbit	Polyclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-tERK	Cell Signaling	Rabbit	Polyclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-pERK	Cell Signaling	Rabbit	Polyclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-HO-1	Cell Signaling	Rabbit	Monoclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-LDHa	Cell Signaling	Rabbit	Monoclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-PCNA	Cell Signaling	Mouse	Monoclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-Keap1	Proteintech	Rabbit	Polyclonal	5% non fat dry milk	1:1000 o/n 4°C
Anti-NRF2	Abcam	Rabbit	Polyclonal	5% non fat dry milk	1:800 o/n 4°C
Anti-rabbit	Cell Signaling	Goat	-	5% non fat dry milk	1:1000 2h RT
Anti-mouse	Cell Signaling	Horse	-	5% non fat dry milk	1:1000 2h RT

Table 2. 2 *Antibody characteristics and formulations*

Antibody	Supplier	Description	Ref #
Anti- β -Actin	Cell Signaling	8H10D10	#3700
Anti-GAPDH	Cell Signaling	14C10	#3683
Anti-CCP-3	Cell Signaling	Cleaved Caspase-3 (Asp175)	#9661
Anti-tAMPK	Cell Signaling	AMP-activated protein kinase	#2532
Anti-pAMPK	Cell Signaling	Phospho-AMPK α (Thr172) (40H9)	#2535
Anti-tERK	Cell Signaling	p44/42 MAPK (Erk1/2)	#9102
Anti-pERK	Cell Signaling	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	#9101
Anti-HO-1	Cell Signaling	Rabbit mAb	#43966
Anti-LDHa	Cell Signaling	Rabbit mAb	#3582
Anti-PCNA	Cell Signaling	Mouse mAb	#2586
Anti-Keap1	Proteintech	kelch-like ECH-associated protein 1 fusion protein Ag0779	#10503-2-AP
Anti-NRF2	Abcam	Recombinant fragment corresponding to Human Nrf2	ab137550
Anti-rabbit	Cell Signaling	HRP-linked Antibody (Conjugated to horseradish peroxidase(HRP))	#7074
Anti-mouse	Cell Signaling	HRP-linked Antibody (Conjugated to horseradish peroxidase(HRP))	#7076

2.4.2 Quantitative Polymerase Chain Reaction (qPCR)

Total mRNA was extracted using the Qiagen RNeasy Mini Kit (74104, Qiagen) as per the manufacture's instruction, and mRNA concentrations were then measured with Qubit assays (Q32852, Invitrogen). The reverse transcription (RT) of 1.0 µg of total RNA (205311, Qiagen) was used to create cDNA and to quantitative polymerase chain reaction (qPCR) analysis. Each 10 µL of PCR reaction mix contained: 5 µL TaqMan fast master mix, 0.5 µL assay mix, 1 µL sample, and 3.5 µL H₂O. Quantitative PCR was used to quantify mRNA expression in samples and assay mixes of NRF2 (Nfe2l2, Rn00477784_m1), Heme-oxygenase-1 (HO-1, Rn01536933_m1), Glutamate-Cysteine Ligase Catalytic Subunit (Gclc, Rn00689046_m1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn01775763_g1) were purchased from ThermoFisher scientific. All samples were examined in duplicate using GAPDH as a housekeeping gene. The qPCR was performed on an ABI prism 7000 machine using the Bio-Rad CFX Connect Real-Time PCR Detection System (40 cycles with 30s annealing at 60 degrees celcius). In the linear range of the amplification, amplification curves were analysed to obtain the cycle threshold (Ct) value. All gene expressions were normalized to the housekeeping gene, and fold change of expressions were calculated using delta value methods (Mean ± SD).

Table 2. 3 *Reagents and volumes used in qPCR*

Reagent	Volume (uL)
Master Mix	10
Probe	
Primer Mix	1
cDNA	9
Rnase-free water	
Final volume	20

Table 2. 4 *qPCR kits*

Reagent	Cat No.
RNA extraction	
Qiagen RNeasy Plus mini Kit	74134
Reverse transcription	
Qiagen Quantitect Reverse Transcription Kit	205311
qPCR	
Taqman Fast Advanced Master Mix	4444557

2.4.3 Hematoxylin and Eosin Staining

Liver samples were fixed with 10% formalin, embedded in paraffin wax and stained with hematoxylin and eosin (H&E) according to standard protocols. Paraffin sections containing liver tissue were cut, placed on glass slides and fixed with methanol for 9 minutes. After drying in room air, sections were first deparaffinized and rehydrated using xylene, ethanol (at 100%, 95% and 80%), each for 3 minutes, followed by deionized water for 5 minutes. Hematoxylin staining was then applied for 3 minutes after which the slides were rinsed in deionised water first followed by regular tap water for 5 minutes. Slides were dipped into (fast) Acid ethanol and rinsed once more with tap water and deionised water. For eosin staining, the slides were dipped in Eosin for 1 minute followed by 95% and 100% ethanol for 5 minutes each and then xylene for a further 5 minutes. A coverslip was then applied before the xylene dried using a glass rod to remove any bubbles.

Biopsies were examined by a histopathologist at our institution who was blinded to sample identity while performing the analysis. Three separate features were specifically examined: the degree of fatty infiltration categorized in macro/microsteatosis, portal triad inflammation using the Banff grading system and IRI parameters as graded by the Modified Suzuki Scoring system [21, 22]. The Banff schema were initially used to grade acute liver allograft rejection and considers portal inflammation, bile duct damage and venous

endothelial inflammation. Each category is graded from 1 to 3 with 3 being the most extensive degree of injury. The criteria were used for the purpose of the current studies to offer an objective and validated scoring system to liver allograft injury (Figure 2.3). The Suzuki scoring system can measure the histopathological degree of hepatic necrosis using a grading system from 0 – 4. This is most commonly used in the assessment of hepatic IRI and categorizes congestion, vacuolization and parenchymal necrosis to assess the severity of liver damage.

Category	Criteria	Score
Portal inflammation	Mostly lymphocytic inflammation involving but not noticeably expanding a minority of the triads	1
	Expansion of most or all of the triads, by a mixed infiltrate contain lymphocytes with occasional blasts, neutrophils, and eosinophils	2
	Marked expansion of most or all of the triads, by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into periportal parenchyma	3
Bile duct inflammation/damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear: cytoplasmic ratio of epithelial cells	1
	Most of the ducts are infiltrated by inflammatory cells. More than occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization of the epithelium	2
	As above for 2, with most of the ducts showing degenerative changes or focal luminal disruption	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltration involving some but not a majority of the portal and/or hepatic venules	1
	Subendothelial lymphocytic infiltration involving most or all of the portal and/or hepatic venules	2
	As above for 2, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis	3

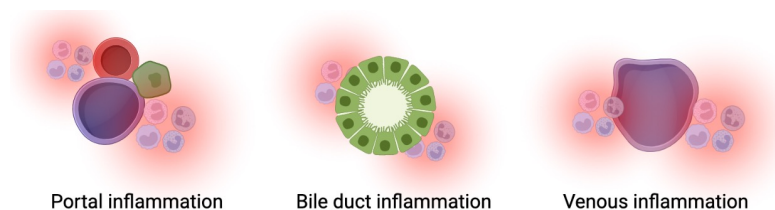
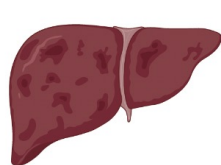


Figure 2. 3 *Banff grading criteria for liver allograft rejection*. This system of injury categorises the degree of inflammatory infiltration and injury to hepatic parenchyma by grading, portal triad, biliary and venous inflammation.

Score	Congestion	Vacuolization	Necrosis
0	None	None	None
1	Minimal	Minimal	Single cell necrosis
2	Mild	Mild	–30%
3	Moderate	Moderate	–60%
4	Severe	Severe	>60%



Congestion



Vacuolization



Necrosis

Figure 2. 4 *Suzuki scoring system for liver ischaemia reperfusion injury*. This is a common system used in liver transplantation literature to assess the degree of IRI in allografts.

2.4.4 Periodic acid-Schiff Staining

To assess hepatic glycogen content, Periodic acid–Schiff (PAS) staining was performed (#395B, Sigma, St. Louis, MO). Tissue sections were fixed with methanol and placed in periodic acid solution for 5 minutes. After washing, sections were submerged into Schiff's reagent for 15 minutes, washed again and counterstained in Gill's Hematoxylin No.3 for 90 seconds. The slides were washed once more, allowed to dry and mounted. Rose or magenta staining with a blue/black nucleus was indicative of PAS positivity and glycogen content. Relative densities were determined using GIMP 2.10.12.

2.4.5 Immunofluorescence staining

Immunofluorescence (IF) staining was conducted to confirm and locate the expression of NRF2 within the hepatic parenchyma and various cell populations using the following antibodies; hepatocytes (anti-HEPPAR1, Novus Biologicals Centennial, CO, USA), macrophages (CST#76437), cholangiocytes (CST#4558S). The evaluation of nuclear NRF2 was performed using the Intensity Ratio Nuclei Cytoplasm tool for ImageJ to determine nuclear translocation following Oltipraz treatment

(SCR_018573; https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Intensity-Ratio-Nuclei-Cytoplasm-Tool). Portal triad inflammation, when present, was determined by assessing the number of CD3+ cells in the portal area using an anti-CD3 antibody (ab16669). A manual cell count was performed by capturing 5 images per sample and manually tagging and counting CD3+ cells within the portal areas. The count is repeated 4 times per image and the average count of all 5 images is determined for each sample. Photomicrographs were taken with a Zeiss Observer Z1 immunofluorescence microscope and images were captured by Axiovision 4.8.2 software.

Table 2. 5 *List of reagents used for Immunofluorescence staining*

Antibody	Supplier	Isotype	Conditions	Cat no
Anti-Keratin 19	Cell Signaling	Mouse	1:200 o/n 4°C	#4558
Anti-Hepatocyte Specific Antigen	Novus Biologicals	Mouse	1:200 o/n 4°C	NBP2- 45272
Anti- CD68/SR-D1	Novus Biologicals	Mouse	1:200 o/n 4°C	NB100- 683
Secondary Antibody				
Goat Anti-Rabbit IgG DyLight® 550	Leinco Technologies	Rabbit	1:200 2h RT	R1322
Goat Anti-Rabbit IgG FITC 488	Leinco Technologies	Rabbit	1:200 2h RT	R114
Goat Anti-Mouse IgG DyLight® 488	Leinco Technologies	Mouse	1:200 2h RT	M1331

2.5 Statistical analysis

Data normality was assessed and parametric tests were used when appropriate. For continuous variables, data were compared using unpaired *t*-tests for normally distributed data and Mann–Whitney *U* test otherwise. Association of categorical variables (differences for dichotomous variables between groups) was assessed using χ^2 test. The distribution of protein expression was observed (Figure 2.5) and NRF2 expression was dichotomized for the purpose of analysis. Similar to previous studies using NRF2 levels, a median split was performed to classify subgroups based on protein expression with immunoblotting [272, 273]. Livers were stratified into a high NRF2 expression group (above the 50th percentile) and a low NRF2 expression group (below the 50th percentile) [247, 274, 275]. Demographic and clinical data were compared to determine their relationship to NRF2 expression. GraphPad Prism 8 software (San Diego, CA) was used to generate graphs and data are presented as mean $\pm$ SD or SEM. For all analyses, $p < 0.05$ was considered statistically significant.

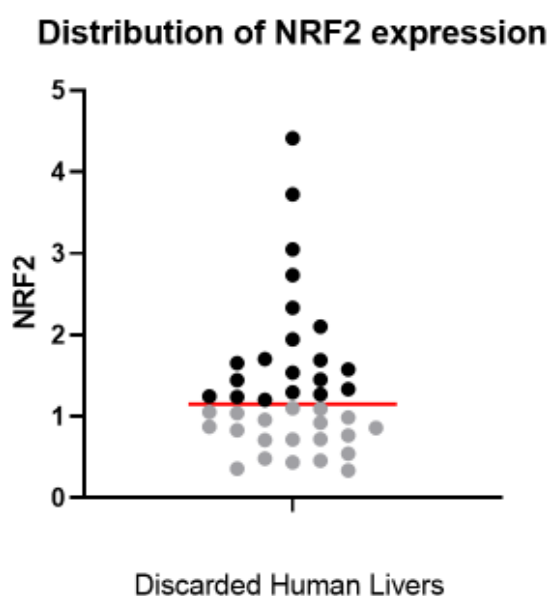


Figure 2. 5 Scatter graph with NRF2 distribution in discarded human livers. Scatter plot of the variable distribution of NRF2 expression in discarded human livers ($n = 40$). Median value = red line.

Chapter 3

The functional expression of nuclear factor erythroid 2–related factor 2 in discarded human liver allografts

3.1 Introduction

The increasing success of liver transplantation (LT) has placed significant pressures on the donor pool and it appears the demand grossly outweighs supply. As the population ages and metabolic conditions become more common, allografts from older donors, steatotic livers and DCDs are becoming more prevalent. To address the deficit in donor livers, there are increasing calls to use these extended criteria donors (ECDs) or marginal organs and expand the donor pool. Historically, the quality of such organs has been called into question because of their increased susceptibility to IRI and marginal allografts have oftentimes led to inferior and unacceptable transplant outcomes [276, 277]. For these reasons, many grafts are discarded each year which can impact national LT utilization [37]. In current practice, up to 45% of retrieved livers from DCDs are discarded due to concerns about viability, many DCD organs are declined without being retrieved and approximately 10% of the liver donor pool faces discard [278].

The safety and quality of marginal livers would be greatly improved by a reliable test or biomarker to quantify the degree of injury and overall condition of the organ. It is likely that some organs are being discarded prematurely for fear of failure and in the current climate of rising liver disease, all efforts to increase the donor pool need to be considered. Hence some discarded livers may still be functional despite meeting current standards for discard. Adding to this contentious issue is the fact that there are no consensus guidelines for discard and most units follow their own arbitrary policies based on broadly accepted guidelines.

The NRF2 axis is known to have important physiologic roles and has multiple effects on redox homeostasis, inflammation, cytoprotection and cell survival processes [279]. Identifying marginal livers with an intact NRF2 axis may help predict the livers ability to withstand IRI and the entire LT process. The rationale for this is that the axis appears naturally downregulated in livers from older donors which ultimately led to less favourable clinical parameters when compared to transplanted allografts from younger donors in a previous study from St Vincent's University Hospital [247]. Supportive data on the expression of NRF2 in marginal livers is currently unavailable and needs to be explored before we can determine whether the pathway can be upregulated in such livers. Assessing

the quality of discarded organs by quantifying their anti-oxidant levels may help identify potentially salvageable livers. Given the putative role of NRF2 in IRI and the increased susceptibility of marginal organs to injury, the pathway may also play a role in the regenerative capacity of an allograft and its ability to recover functionally. Mechanisms underlying IRI in steatotic livers, in particular, appear to be related to their altered energy homeostasis [280]. Additionally, fatty livers appear predisposed to rapid necrotic cell death and endoplasmic reticulum stress mediating further inflammation and injury [281]. The presence of similar homeostasis responses in other marginal organs is worth exploring and how these may relate to the NRF2 pathway. Although the NRF2 pathway has traditionally been considered as a cytoprotective response, its regenerative ability through alternative metabolic and degenerative pathways may provide insight into the functional evaluation of marginal allografts.

Hypothesis:

The NRF2 axis influences allograft quality in liver transplantation and may provide a rational biomarker for allograft selection and therapeutic targeting.

Overall aim:

To assess the link between hepatic NRF2 expression and allograft function, injury and repair systems.

3.2 Specific aims

The aims of this chapter include:

- Assessing the expression of NRF2 in discarded human livers.
- Investigating the functional role of NRF2 on liver allograft quality and injury.
- Exploring the molecular impact of NRF2 on discarded livers.

3.3 Results

3.3.1 NRF2 expression in discarded human livers and donor characteristics

Western blotting analysis revealed that hepatic NRF2 expression was highly variable in the 40 donor livers (median age 42 years, range 13–61; male n =25; median relative expression =1.16, range 0.33 – 4.41). The median donor BMI was 29.3 kg/m² (range 14.3 kg/m² -50.3 kg/m²) and 20 patients met the criteria for moderate (n =9), severe (n =9) or very severe (n =2) obesity. Anoxia was the most common cause of donor death (n =21), followed by cerebrovascular accidents (n =11) and trauma (n=8). Livers were most often discarded for exceeding Donor after Cardiac Death (DCD) times (n =18), the inability to identify a suitable recipient (n =9), hepatic steatosis (n =7), advanced donor age (n =5) and fibrosis (n =1). Livers were then divided into a high NRF2 expression group and a low NRF2 expression group, as previously done by Zaman and colleagues[247], to assess for any relevant and associating characteristics; mean protein expression in the high NRF2 group was 1.91 ± 0.88 and mean protein expression in the low NRF2 group was 0.81 ± 0.27 (Table 3.1). Donor age was comparable in both groups; high NRF2 group median age 43 (range 13 – 61) compared to low NRF2 group median age 40 (range 14 – 61); p = 0.079. Additionally, donors with lower levels of NRF2 tended to have a higher and obese BMI approaching statistical significance when compared to donors with lower expressions of hepatic NRF2; low NRF2 group mean BMI 27 ± 7.8 kg/m² compared to high NRF2 group mean BMI 32 ± 7.4 ; p = 0.91. Two of the livers in the high NRF2 group were from DBD donors and 4 of the livers from the low NRF2 group were from DBD donors. Nearly a third of livers from the high NRF2 group (n = 12; 30%) were declined and discarded for exceeding DCD warm ischaemia times (mean 34 min $\pm$ 18 minutes), 3 livers were discarded for histological steatosis and fibrosis and one liver was discarded due to the advanced age of the donor (> 65 years). In the low NRF2 expression group, only 6 livers were declined and discarded for exceeding DCD warm ischaemia times, an additional 5 were declined for histological evidence of steatosis and fibrotic changes on the allograft and 4 livers were discarded due to the advanced age of the donor. A total of 9 livers were discarded because of the inability to identify a suitable donor in a timely fashion. Although these were considered acceptable for transplantation at the time, on protein quantitation, 4 of these livers were in the high NRF2 group and 5 were in the low NRF2 group.

Table 3. 1 *Demographic details of the donor population in the high NRF2 and low NRF2 expression groups.*

Variables	High NRF2 expression	Low NRF2 expression
N	20	20
Gender (M:F)	16:4	15:5
Age, median (range)	43 (13 - 61)	40 (14 - 61)
Race		
Caucasian	16	17
African American	4	2
Asian	-	1
BMI, mean (SD)	27 (7.8)	32 (7.4)
Lean : steatotic	14:6	8:12
WIT, minutes (SD)	34 (18)	37 (25)
Donor cause of injury		
Anoxia	11	10
CVA	4	7
Trauma	5	3
Reason for organ discard		
Exceeded DCD time	12	6
No eligible recipient identified	4	5
Steatosis/Fibrosis	3	5
Advanced donor age	1	4

3.3.2 NRF2 expression on hepatic allograft quality and injury

Donor livers were divided into two groups based on high NRF2 (mean expression 1.91 ± 0.88) and low NRF2 (mean expression 0.81 ± 0.27) levels and specific allograft characteristics were once again observed. No significant differences were observed in warm ischaemia times (WIT) between DCD livers with high NRF2 expression (mean 34 ± 18 minutes) and the low NRF2 group (37 ± 25 minutes; $p = 0.31$). Donor serum analyses were compared 24 hours before liver procurement. Pre-procurement mean donor serum alanine transaminase (ALT) was significantly lower in the high hepatic NRF2 expression group (53 ± 58 u/L vs 152 ± 228 u/L; $p = 0.049$) compared to the low NRF2 liver group. Similarly, aspartate transaminase (AST) enzyme levels were significantly lower pre-operatively (58 ± 46 u/L vs 200 ± 100 u/L; $p = 0.045$) in donors categorized in the high NRF2 group (Figure 3.1). Serum glucose levels were decreased in the high NRF2 expression group (129 ± 47 mg/dL vs 171 ± 81 mg/dL; $p = 0.049$) pre-procurement while serum albumin levels were significantly increased (2.7 ± 0.43 g/dL vs 2.4 ± 0.5 g/dL; $p = 0.027$).

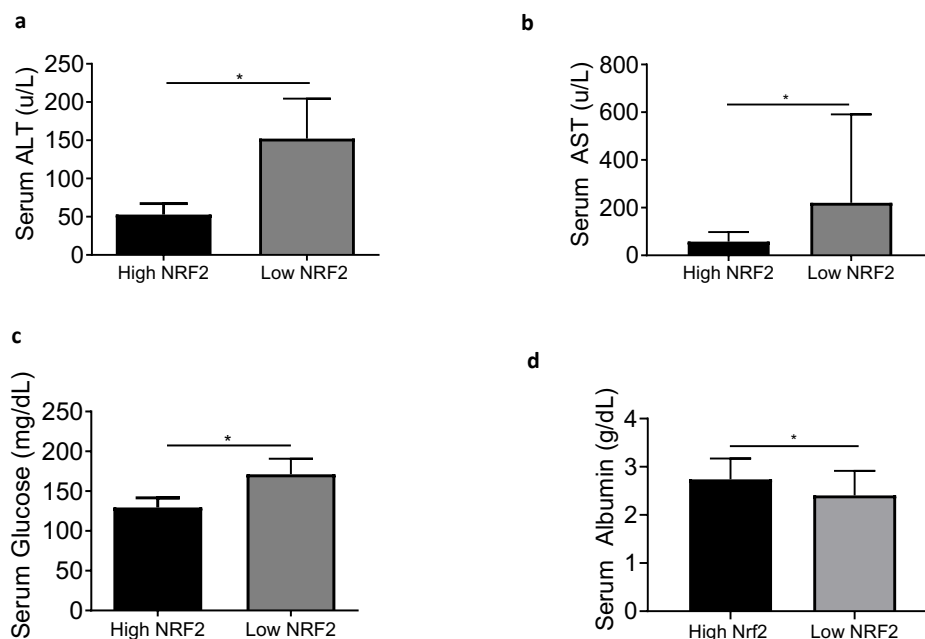


Figure 3. 1 *Biochemical results in livers from high and low NRF2 expression donors (n = 40).*

Livers with high NRF2 expression demonstrated significantly lower a) alanine aminotransferase (ALT), b) aspartate aminotransferase (AST), and c) glucose but higher serum albumin levels. Data are represented as mean $\pm$ SD. *, $p < 0.05$ by t-test.

Discarded liver histopathological analysis on H & E staining revealed a lower percentage of small droplet macrosteatosis in livers with high NRF2 levels (mean 7.6% steatosis) when compared to livers with lower hepatic protein expression of NRF2 (mean 17% steatosis; $p = 0.04$). Comparable degrees of large droplet macrosteatosis were observed in the high NRF2 group (mean 8.5% steatosis) when compared to the low NRF2 group (17.5% steatosis). Histopathological characteristics and inflammatory scores are detailed in Table 3.2.

Donor livers with lower NRF2 levels had increased vascular inflammation (Figure 3.2), manifesting as increased inflammatory cell infiltration of the hepatic artery and portal vein (hepatic artery: high NRF2 expression group mean inflammatory Banff score 1.05 vs 1.5 in the low NRF2 expression group; $p = 0.01$; portal vein: high NRF2 expression group mean inflammatory Banff score 1 vs 1.45 in the low NRF2 expression group; $p = 0.003$) (Figure 3.3). On IF, increased periportal CD3 positivity was observed in low NRF2 livers ($p < 0.001$) (Figure 3.4) when compared to livers with low levels of NRF2 expression indicating a greater degree of inflammatory cell infiltration in low NRF2 environments. On PAS staining, lower hepatic glycogen was also observed in livers with high NRF2 expression levels when compared to livers with low NRF2 levels ($p = 0.046$) (Figure 3.5).

Table 3. 2 *Histological analysis of discarded high and low expressed NRF2 livers*[282]

	Low NRF2 Expression (n = 20)	High NRF2 Expression (n = 20)	P value
Large droplet macrosteatosis % mean (range)	17.5 (0-70)	8.5 (0-70)	0.21
Small droplet macrosteatosis % mean (range)	17 (0-60)	7.6 (0-50)	0.04
Inflammatory score			
Suzuki Scoring			
Necrosis	0.1	0.35	0.23
Vacuolization	1.3	1.8	0.21
Congestion	0	0	-
Banff Criteria			
Bile duct inflammation	1.7	1.5	0.29
Vascular inflammation			
Hepatic artery	1.5	1.05	0.01
Hepatic vein	0.25	0.41	0.52
Portal vein	1.45	1	0.003
Peri-portal inflammation	1.7	1.5	0.32

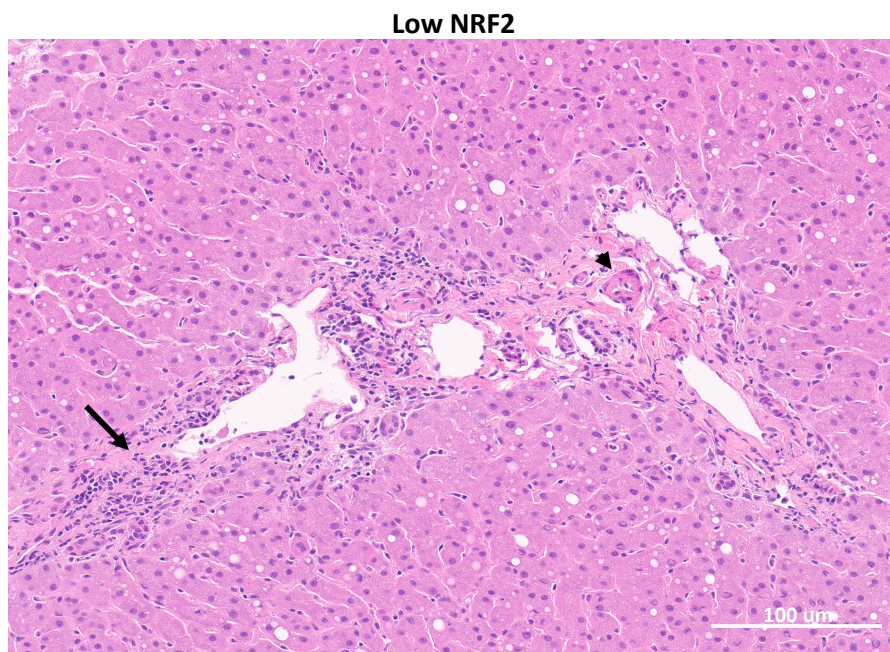
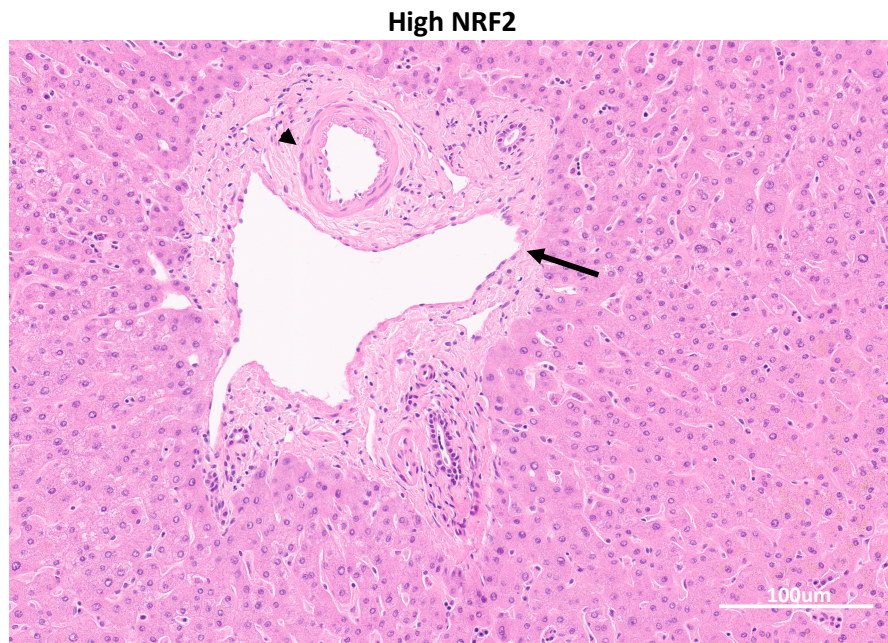


Figure 3. 2 Inflammation in representative hematoxylin and eosin staining of discarded donor livers. Livers in the high NRF2 group had minimal hepatic artery (arrow heads) and portal vein (arrows) inflammation when compared to livers from the low hepatic NRF2 expression group which demonstrated greater inflammatory cell infiltration. There is more architectural disturbance around the portal triad in the low NRF2 group and increased clusters of inflammatory cell nuclei.

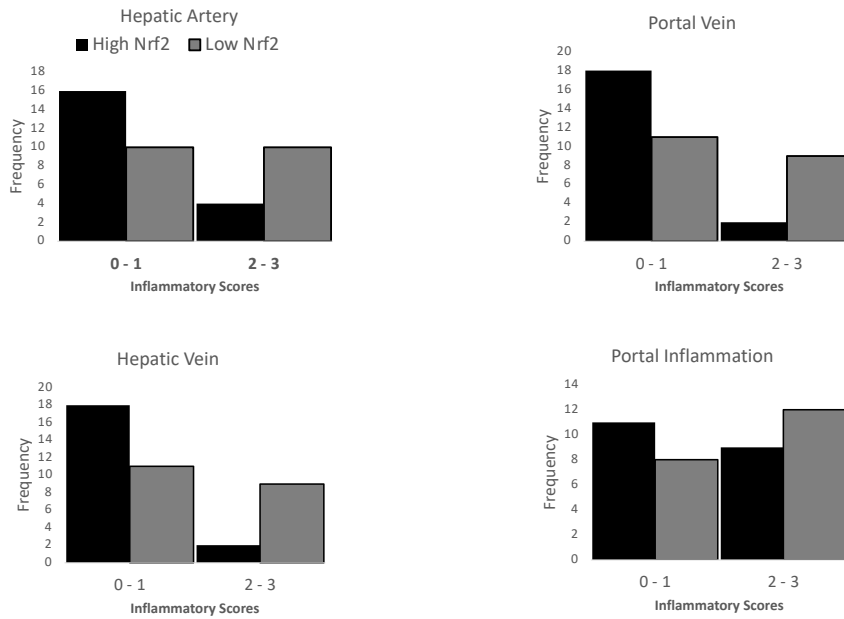


Figure 3. 3 Histograms representing histological inflammatory scores. Livers with higher expressions of NRF2 (n = 20) had lower inflammatory scores and significantly lower hepatic artery ($p = 0.01$) and portal vein inflammation ($p = 0.003$). Most of the low hepatic NRF2 expression donor livers (n = 20) fell into higher histological inflammation categories.

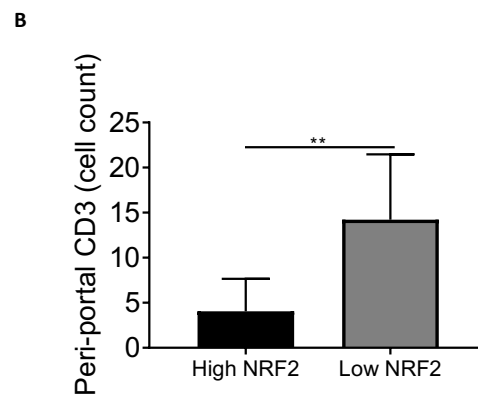
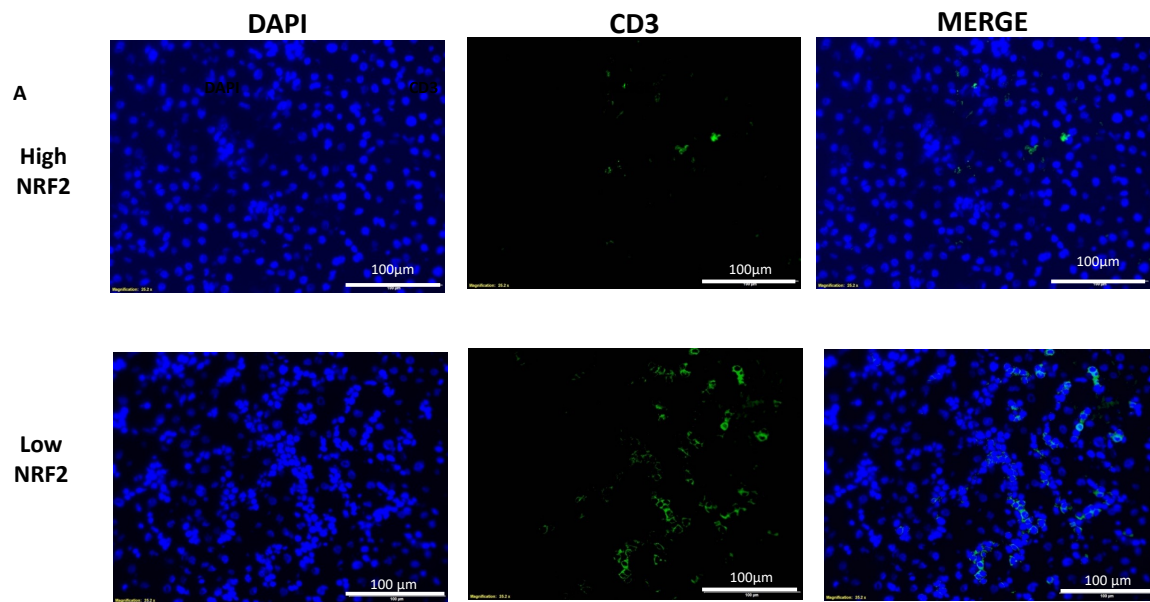


Figure 3. 4 Immunofluorescence staining for T cells (CD3, green) and nuclei (DAPI, blue) of high and low NRF2 groups from discarded human livers (n = 40). Quantitation of immunofluorescence staining for CD3⁺ cells around the portal triad was performed in both high and low NRF2 groups. This indicated increased **A)** CD3⁺ positivity and inflammatory cell infiltration around the portal triad in livers from the low NRF2 group when compared livers with higher expression of NRF2 with **B)** manual cell counting. Data are represented as mean $\pm$ SD. *, p<0.05, **, p<0.01 by t-test.

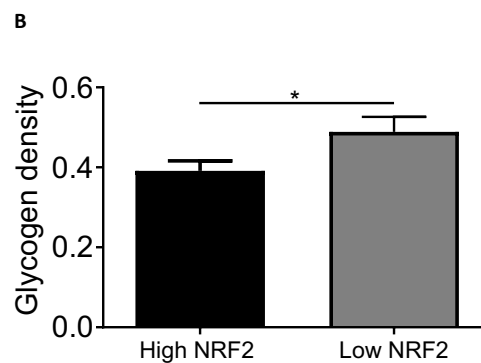
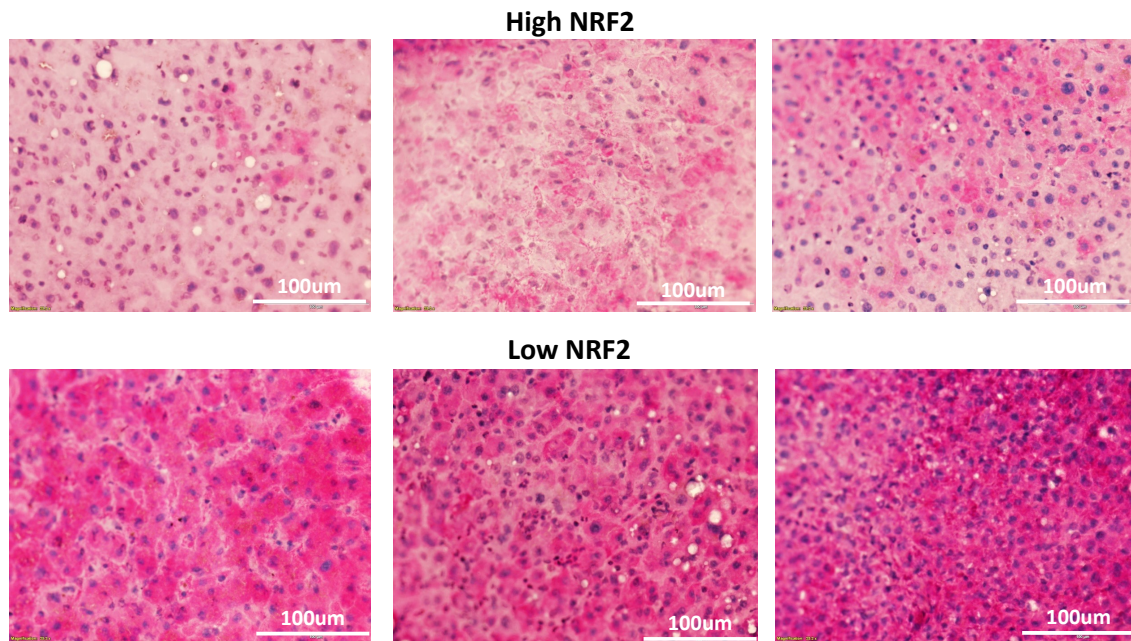


Figure 3. 5 Representative micrographs from Periodic-acid Schiff staining (PAS) of discarded human livers from high and low NRF2 groups. **A)** On microscopic examination of liver specimens, livers with higher hepatic NRF2 expression demonstrated less glycogen storage on PAS staining when compared to livers with lower hepatic NRF2 expression. **B)** Quantitation of relative intensity of glycogen content revealed higher glycogen content in livers with lower expression of hepatic NRF2. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

3.4 Molecular impact of NRF2 on discarded livers

3.4.1 Hepatic NRF2 and the AMP-activated protein kinase (AMPK) pathway

Molecular alterations in high and low NRF2 livers were then assessed on western blot and densitometric analysis to explore the potential interplay between antioxidant and metabolic mechanisms. The AMP-activated protein kinase (AMPK) pathway was explored by evaluating phosphorylation levels in high NRF2 and low NRF2 liver groups and standardizing its expression to total AMPK levels (Figure 3.6). Expression varied widely across samples (median = 0.946, range 0.299 – 4.7).

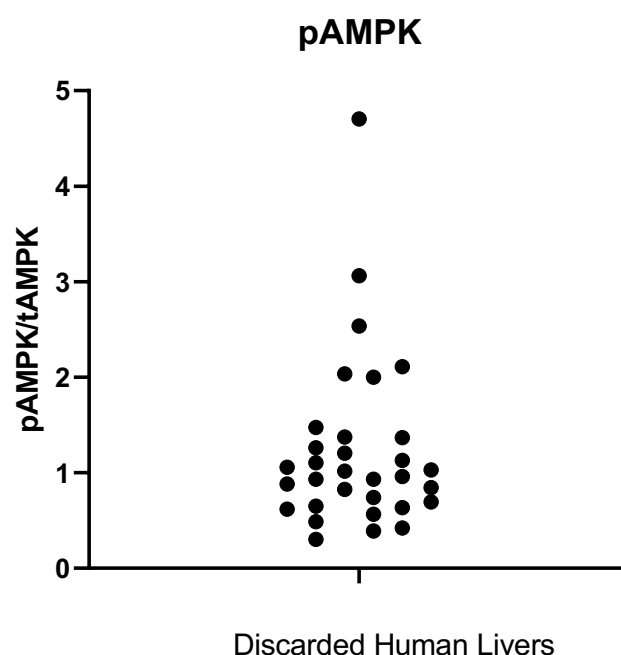


Figure 3. 6 Scatter graph of pAMPK protein expression based on western blotting and distribution in the sample of discarded human liver allografts (n = 40). There was a wide variation in expression levels across the included samples.

Livers with high expressions of NRF2 demonstrated increased AMPK phosphorylation when compared to livers with lower hepatic expression of NRF2 and this was statistically significant (1.51 ± 1.1 versus 0.89 ± 0.54 , $p = 0.045$). Although total AMPK was higher in livers with higher NRF2 expression when compared to livers with lower NRF2 expression

levels, this did not reach statistical significance (1.65 ± 1.41 versus 1.15 ± 0.64 , $p = 0.14$) (Figure 3.7).

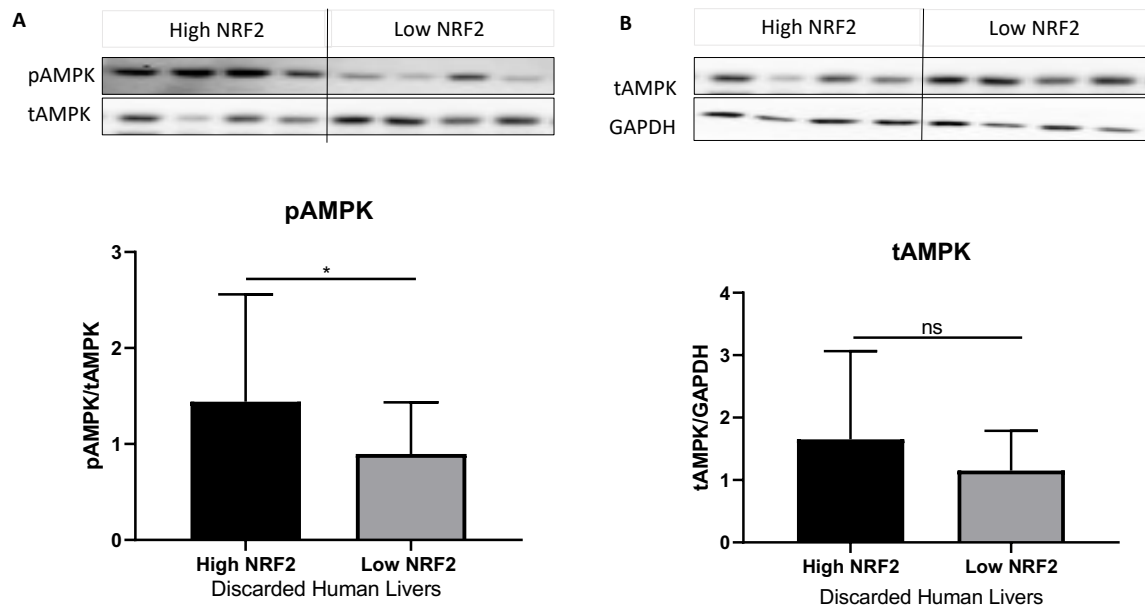


Figure 3. 7 Western blot analysis of phosphorylated and total AMP-activated protein kinase in high (n = 20) and low expression NRF2 livers (n = 20). A) Livers with higher hepatic expression of NRF2 demonstrated higher protein expression levels of pAMPK in hepatic tissue. Total AMPK protein expression was comparable in both high and low NRF2 expression livers. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

3.4.2 Hepatic NRF2 and Heme oxygenase-1 (HO-1)

Downstream anti-oxidant activity was examined by determining heme-oxygenase-1 (HO-1) expression in the studied samples and the previous literature indicated that this was the most commonly measured gene in the NRF2 pathway. Once more, a wide variation of HO-1 expression (median 0.767; range 0.10 – 1.34) was observed among the discarded liver samples (Figure 3.8). Livers with higher expression levels of NRF2 had increased levels of hepatic HO-1 when compared to livers with low hepatic NRF2 levels and values were approaching significance (High NRF2 expression livers 0.61 ± 0.31 versus low NRF2 expression livers 0.36 ± 0.31 , $p = 0.06$) (Figure 3.9).

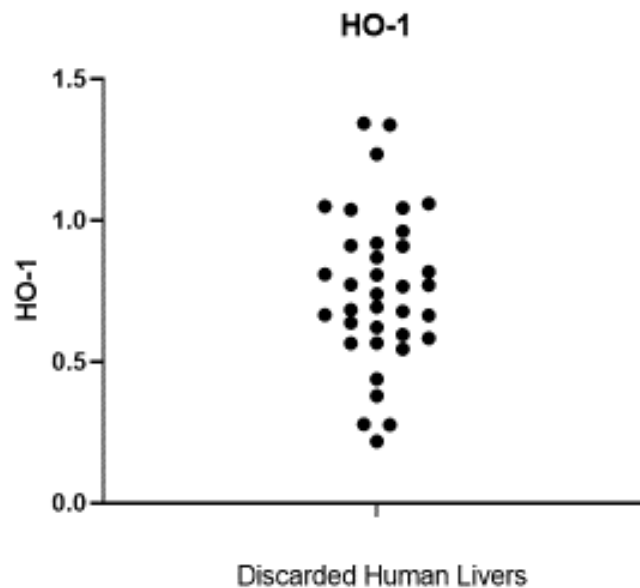


Figure 3. 8 Scatter graph of HO-1 expression and distribution in the sample of discarded human liver allografts (n = 40). There was a wide variation in expression levels across the included samples.

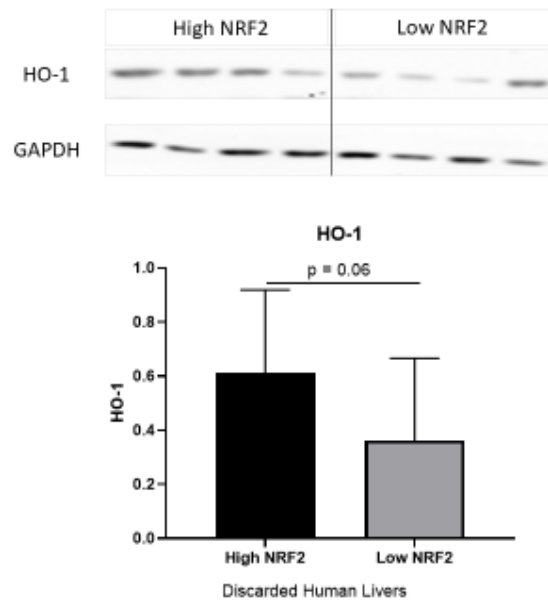


Figure 3. 9 Hepatic expression of Heme Oxygenase-1 (HO-1) in relation to NRF2 expression levels. Although livers with higher expression levels of NRF2 (n = 20) demonstrated higher HO-1 expression, this did not reach statistical significance when compared to livers with lower expression levels of hepatic NRF2 (n = 20). Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

3.4.3 Hepatic NRF2 and Kelch-like ECH-associated protein 1 (Keap 1)

It was hypothesized that the inhibitory protein Keap 1 was decreased in livers with higher hepatic NRF2 expression and Keap 1 protein expression was assessed on immunoblotting. As with other markers, wide variation was observed in hepatic Keap 1 expression among the studied discarded human livers (median = 1.918, range 0.536 – 13.44) (Figure 3.10).

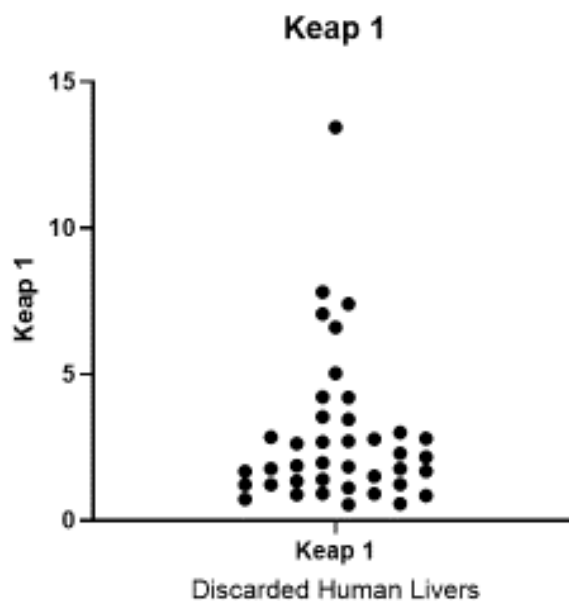


Figure 3. 10 Scatter graph of Keap1 expression and distribution in the sample of discarded human liver allografts (n = 40). There was a wide variation in expression levels across the included samples.

Although there were marginally lower Keap 1 protein expression levels in livers with higher NRF2 expression when compared to livers with low NRF2 expression, these differences did not reach statistical significance (High NRF2 expression livers 1.13 ± 0.09 versus low NRF2 expression livers 1.19 ± 0.07 , $p = 0.07$) (Figure 3.11).

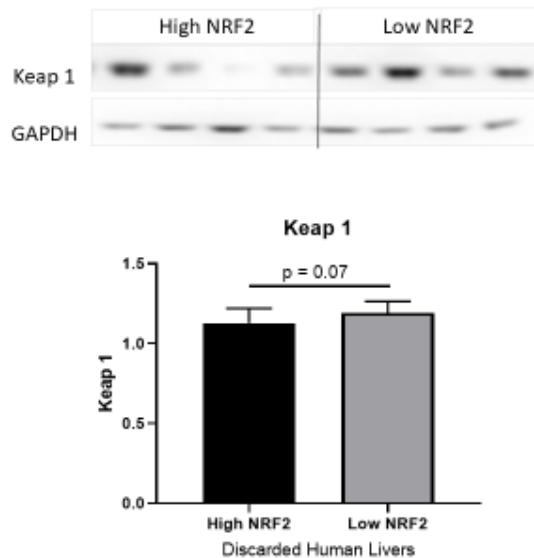


Figure 3. 11 Hepatic expression of Keap 1 in relation to NRF2 expression levels. Although livers with higher protein expression levels of NRF2 ($n = 20$) demonstrated lower Keap 1 expression, this did not reach statistical significance when compared to livers with lower expression levels of hepatic NRF2 ($n = 20$). Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

3.4.4 Hepatic NRF2, cell proliferation and survival signaling

To further evaluate the relationship between NRF2 and cell proliferation, differences in Extracellular Signal-regulated Kinase-1 (ERK) phosphorylation were evaluated in both high and low NRF2 expression liver groups. Protein expression on immunoblotting varied widely (median = 0.226, range 0.0299 – 4.529) (Figure 3.12). Higher expression levels of phosphorylated ERK was observed in the high NRF2 group (2.53 ± 1.65 vs 1.39 ± 0.49 , $p = 0.041$) (Figure 3.13).

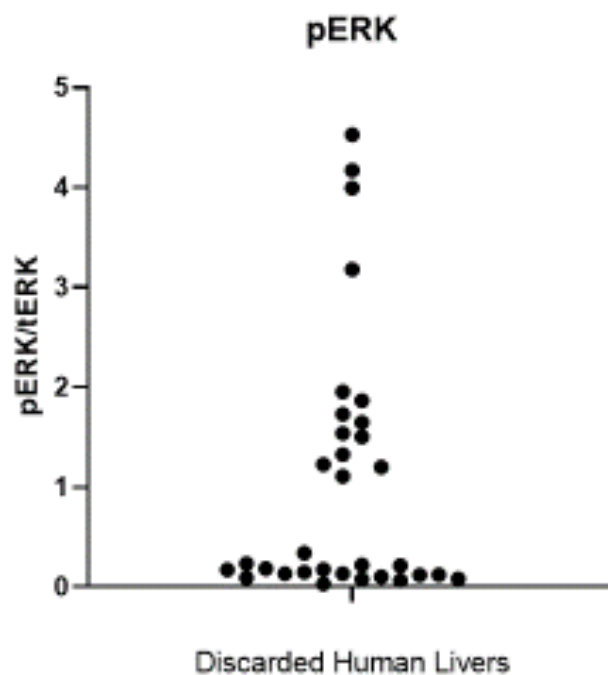


Figure 3. 12 Scatter graph of pERK expression and distribution in the sample of discarded human liver allografts (n = 40). There was a wide variation in expression levels across the included samples.

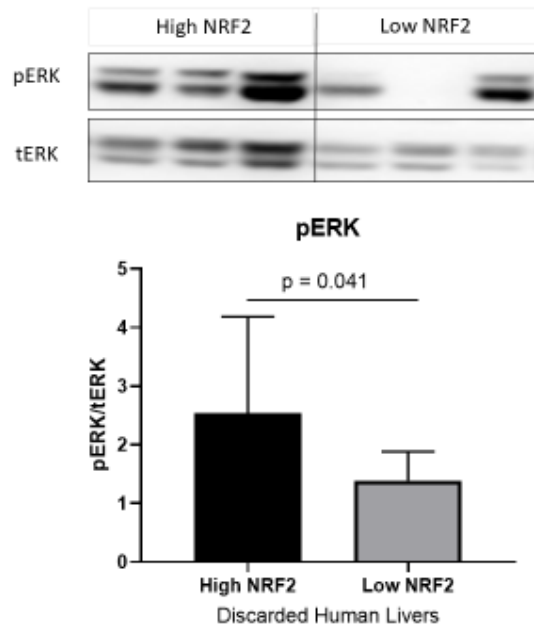


Figure 3. 13 Hepatic expression of pERK in relation to NRF2 expression levels in discarded human liver allografts (n = 40). Livers with higher expression of hepatic NRF2 demonstrated significantly higher protein expression levels of pERK in hepatic tissue when compared to livers with lower hepatic NRF2 expression. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

To capture cell death and apoptotic signalling, the cleaved caspase 3 (CCP-3) expression in both groups was assessed and no differences were observed between the high and low NRF2 group (3.37 ± 2.01 versus 3.7 ± 3.7 , $p = 0.41$) (Figure 3.14).

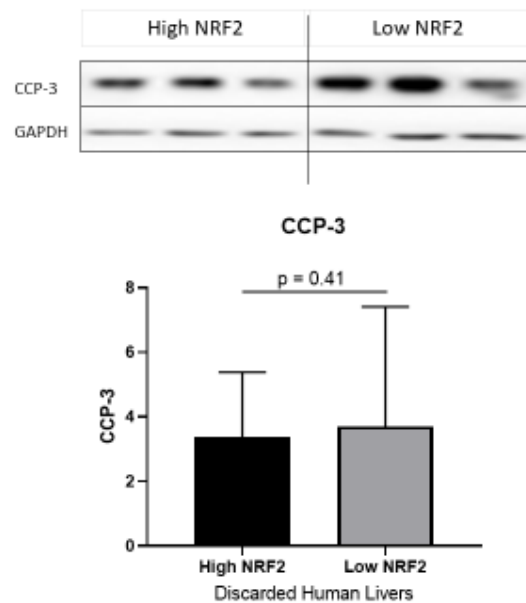


Figure 3. 14 Discarded liver expression of CCP-3 in relation to NRF2 protein expression levels on western blotting. Protein expression levels of CCP-3 were comparable in both the high ($n = 20$) and low ($n = 20$) NRF2 expression livers. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

The expression of LDH α was also determined in discarded human livers and was found to be higher in livers with higher NRF2 expression, although this did not reach statistical significance (High NRF2 expression livers 1.34 ± 0.65 versus low NRF2 expression livers 1.05 ± 0.794 ; $p = 0.225$) (Figure 3.15).

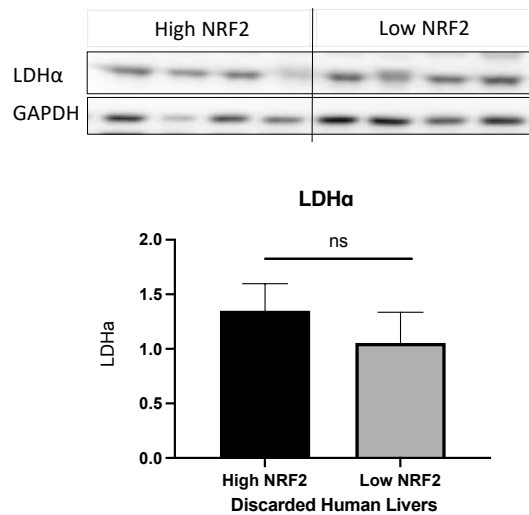


Figure 3. 15 Hepatic expression of LDH α in relation to NRF2 expression levels. There were no differences on western blotting protein expression levels of LDH α which were comparable in both the high and low NRF2 expression livers. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

3.5 Hepatic NRF2 and steatosis

Hepatic steatosis is defined when more than 5% of hepatocytes have microscopic features of fatty droplets within the cell [283]. In total, steatotic livers accounted for 45% (n = 18) of the total donor group and NRF2 status was once more analyzed and compared in both groups of livers. Western blotting analysis demonstrated that lean livers expressed significantly higher NRF2 levels (lean livers relative mean NRF2 expression 1.74 ± 1.02 vs steatotic livers NRF2 expression 0.97 ± 0.36 ; $p = 0.002$) when compared to steatotic samples (median steatosis 25% [range 10 –70%]) (Figure 3.16a). Unsurprisingly, HO-1 expression levels were also significantly lower in steatotic liver samples when compared to lean livers. Hepatic steatosis was then graded from 0% to 70% to reflect the studied population sample and NRF2 levels were again assessed. On densitometric analysis of samples, NRF2 expression was markedly decreased beyond 40% hepatic steatosis except in one liver sample with 70% steatosis which still maintained a strong expression level (Figure 3.16b). The hepatic expression of HO-1 followed a similar pattern, appearing to decrease after 40% steatosis. The same liver with 70% steatosis did not appear to express HO-1 as strongly, however, HO-1 was still observed in the second liver with 70% steatosis, despite having a low expression level of NRF2. Unsurprisingly, livers with higher degrees of steatosis demonstrated higher inflammatory scores (Table 3.3). Hepatic inflammation was then compared in livers with greater than or less than 40% steatosis and, unsurprisingly, livers with higher degrees of steatosis demonstrated higher inflammatory scores. Hepatic Keap 1 expression, although varying significantly across liver samples, was higher in steatotic allografts and trending towards statistical significance (0.84 ± 0.23 versus 0.66 ± 0.29 , $p = 0.051$).

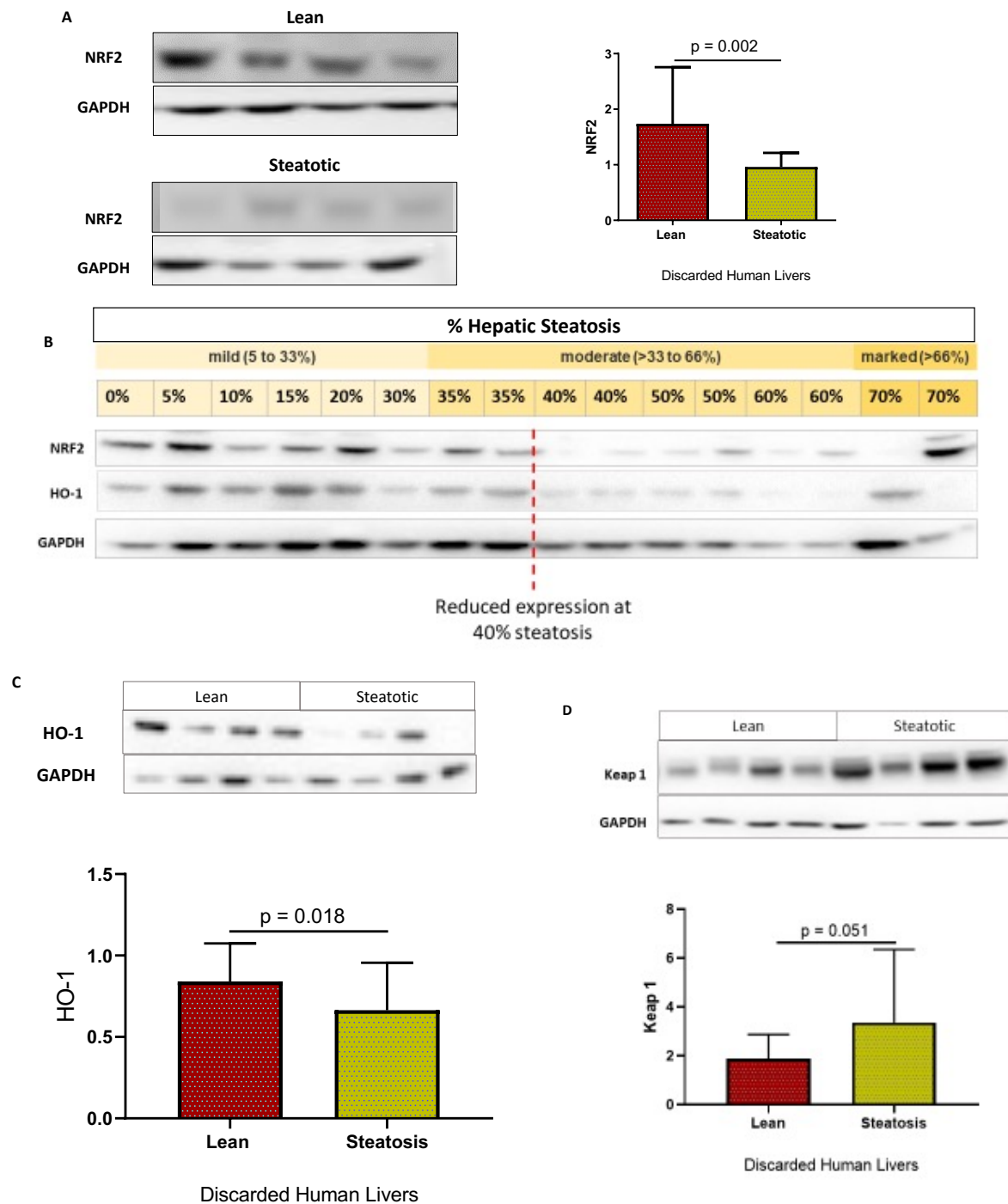


Figure 3. 16 Hepatic NRF2 expression in lean and steatotic liver donors. **A)** Lean liver (n = 22) samples demonstrated higher NRF2 expression levels when compared to steatotic livers (n = 18). **B)** When steatosis was graded, it was apparent that NRF2 levels markedly decreased when more than 40% hepatic steatosis (n = 10) was present. **C)** HO-1 expression was also significantly lower in steatotic liver samples (n = 18) compared to lean livers (n = 22). **D)** Keap -1 was marginally increased in steatotic livers when compared to lean livers. Data are represented as mean $\pm$ SD. *, $p < 0.05$, **, $p < 0.01$ by t-test.

Table 3. 3 Histological features of livers with steatosis greater than 40%

	< 40% steatosis (n = 8)	≥ 40% steatosis (n = 10)	P value
Necrosis	0	0.5	0.28
Bile duct inflammation	1.75	2.2	0.46
Vascular inflammation			
Hepatic artery	1.3	1.7	0.39
Hepatic vein	0.5	0.3	0.65
Portal vein	1.5	1.3	0.65
Vacuolization	1.3	1.8	0.38
Peri-portal inflammation	1.5	1.8	0.31
Portal CD3 cell count/field	6	10	0.07

High and low NRF2 groups were compared once more for relative biochemical analyses. Out of the 20 high NRF2 liver group, 6 livers were categorised as steatotic and the high NRF2 steatotic group was then compared to the high NRF2 lean liver group. Serum ALT (46 ± 39 vs 58 ± 64 ; $p = 0.34$), AST (49 ± 39 vs 57 ± 40 ; $p = 0.36$), glucose (129 ± 37 vs 140 ± 61 ; $p = 0.36$) and albumin (2.6 ± 0.4 vs 2.8 ± 2.43 ; $p = 0.18$) were all comparable. Among the low NRF2 liver group, 12 livers were steatotic and, once more, biochemical analyses of the low NRF2 steatotic group were compared to those of the low NRF2 lean group. Although low NRF2 steatotic livers demonstrated a 2-fold increase in serum enzyme levels, this was not statistically significant (low NRF2 steatotic livers: serum ALT 190 ± 262 IU; serum AST 266 ± 437) when compared to lean livers with low expression levels of NRF2 (low NRF2 steatotic livers: serum ALT 73 ± 86 IU; serum AST 102 ± 115) ($p > 0.1$). Similarly, serum glucose (144 ± 49 mg/dL vs 190 ± 100 mg/dL; $p = 0.08$) and albumin levels (2.5 ± 0.58 g/dL vs 2.6 ± 0.62 g/dL; $p = 0.2$) did not differ significantly. Lean livers with high expression levels of hepatic NRF2 ($n = 14$) were then compared to lean livers with lower expression levels of NRF2 ($n = 8$). Interestingly, leaner high NRF2 expression livers demonstrated lower serum enzyme analyses, however, significance was not reached (serum ALT 58 ± 64 IU vs 73 ± 86 IU; $p = 0.32$, serum AST 58 ± 41 IU vs 102 ± 115 IU; $p = 0.12$) and, similarly, no differences were seen in serum glucose (140 ± 61 mg/dL vs 191 ± 100 mg/dL; $p = 0.08$) or serum albumin (2.8 ± 0.43 g/dL vs 2.6 ± 0.62 ; $p = 0.2$). Steatotic livers with higher expression levels of NRF2 were then compared to steatotic livers with lower levels of hepatic NRF2 and serum ALT (46 ± 39 IU vs 190 ± 262 IU; $p = 0.10$), AST (49 ± 39 IU vs 266 ± 437 IU; $p = 0.12$), glucose (130 ± 37 mg/dL vs 144 ± 49 mg/dL; $p = 0.27$) and albumin levels (2.6 ± 0.41 g/dL vs 2.5 ± 0.58 g/dL; $p = 0.33$) did not differ. Steatotic livers with higher expression levels of NRF2 were also compared to lean livers with lower expression of NRF2. Serum ALT (46 ± 39 IU vs 190 ± 262 IU; $p = 0.24$), AST (49 ± 39 IU vs 102 ± 115 IU; $p = 0.15$), glucose (130 ± 39 mg/dL vs 190 ± 100 mg/dL; $p = 0.09$) and albumin (2.6 ± 0.41 g/dL vs 2.6 ± 0.62 g/dL; $p = 0.49$) did not differ.

3.6 Discussion

Despite an increase in liver transplant volume in recent years, there is still a disparity between organ availability and clinical need. In the US in 2018, up to 30% of patients awaiting liver transplant did not receive an allograft and similar statistics were observed in European and UK data where over 20% of patients on the wait-list will not access a liver [26, 284, 285]. More than 20% of liver allografts from deceased donors, intended for transplantation, are discarded while less than 40% of DCD livers are actually transplanted [46, 286]. As such it has been repeatedly proposed that discarded allografts are the most accessible organ source for transplantation and more aggressive transplant approaches with the increased use of marginal allografts are warranted to decrease death on the wait list [46, 74, 287].

The current chapter aimed to determine the NRF2 profile of 40 discarded human liver allografts and initially revealed a wide variation of NRF2 environments within livers. These findings indicate that some donors might be better at “activating” the pathway and raises the possibility of genetic influences on the tolerogenic properties of livers. Genetic variations on immune responses during liver transplantation have been previously described which may help divide allografts into genetically high and low-risk organ donations [288, 289]. A recent study described major complement allelic variations of C3 among different populations and the frequency of certain recipient genotypes appeared to influence first year patient survival post-transplant [290]. Although protein expression was considered for these experiments, there are potential limitations in relation to demonstrating overall functional expression. Future methodology can apply electrophoresis mobility shift assay (EMSA) or plasmid transfection techniques to assess and quantify NRF2 protein. The quality of the frozen tissue samples may still impact results and these would still have to be interpreted with caution. Nevertheless, the current experiment provide a reliable measure of protein content in discarded human livers. When livers were dichotomized into a high NRF2 expression group and a low NRF2 group in the current experiments, the impact of NRF2 on hepatic function became more apparent and the results indicated the advantage of a higher NRF2 environment.

Donor age, BMI and warm ischaemia times were comparable in both high and low NRF2 livers and, although this could be related to the low sample number, it helped minimise the external impact of potential confounders in the relationship of NRF2 and discarded livers. In a study of 14 donor livers, Zaman and colleagues were one of the first papers to demonstrate the expression of NRF2 in human donor livers and in their analysis linked younger donor age to higher NRF2 expression [247]. Although this specific association was not demonstrated here, previous evidence supports the hypothesis that ageing leads to a decline of NRF2 and results in greater oxidative injury. Shih and colleagues assessed the expression of antioxidant enzymes in rat livers of various ages ranging from (2, 12 and 18–24 months old) and concluded that erythrocyte hemolysis induced by free radicals increased in an age dependant manner [291]. This finding mirrored the decrease in antioxidant enzyme activity with increasing rat age and, consequently, higher levels of oxidative stress. A similar observation was made by Suh et al in their study of livers from older (24–28 months) and younger (2–5 months) rats. The authors observed a 50% age related loss in both total and nuclear NRF2 levels. Interestingly, the authors then attempted to determine whether the constitutive loss of NRF2 also impacted the inducible impact of the gene and its translocation. Using (R)- α -lipoic acid to treat older rats, there was an increase in nuclear NRF2 levels which translated to a similar increase in the downstream activity of GCLC and GCL and attenuating antioxidant losses [292]. The link between NRF2 and a high BMI specifically remains controversial despite a clearer relationship between NRF2 and hepatic steatosis. The association with obesity is confounded by the pro- inflammatory state of obese individuals. Obesity has been shown to lead to an abnormal secretion of adipokines and lipokines by adipocytes and immune cells in adipose tissue, contributing to a constant state of oxidate stress and chronic inflammation[293]. Some of the studies on obesity and NRF2 are contradictory. In in some instances, NRF2-KO mice were protected from high fat diet induced obesity [294], inconsistent with other studies which appeared to show NRF2 activation suppressing similar diets and protecting livers from lipid deposition [295]. In the current context of discarded livers, true correlations could not be accurately construed. Additionally, although prolonged warm ischaemia times are known to worsen allograft function and IRI, no differences were seen in high and low NRF2 livers in the studied discards. Different modes of donor death may result in different degrees of NRF2 activation and in this context, the impact of WIT in DCD donation should be a focus for future

research. Kim et al assessed the effects of WITs on hepatocyte membrane bile transporters as an indicator of IRI and subsequent cholestasis [296]. The authors found that bile transporter expression did not increase in NRF2-KO mice after a period of short warm ischaemia which resulted in worse hepatic injury during IRI. Additional studies investigating the impact of prolonged ischaemia times are warranted in the context of IRI, although the relatively short half-life of the gene may make these investigations challenging [297].

Discarded livers with higher levels of NRF2 demonstrated less injury, manifesting as low transaminases on serum analyses and reduced histological inflammation supporting the hypothesis that NRF2 confers anti-inflammatory properties. Previous research has considered cell membrane instability and leakage as the main driver for higher serum transaminases in hepatic injury [298]. In oxidative stress, ROS accumulation leads to a more oxidizing environment, affecting cellular homeostasis and leading to consequential protein, lipid and membrane damage [299]. In environments that lack antioxidant defence mechanisms, such as the low NRF2 livers, alterations in plasma membrane structures and permeability may contribute to enzyme release as seen in the current set of experiments. Specifically, protein oxidation has been shown to inactivate membrane bound enzymes such as adenosine triphosphatase and glucose-6-phosphatase altering membrane structures and functions [300]. As a consequence, this causes the drop in membrane potential and increased ion permeability of cell contents. Additionally, higher levels of hepatic NRF2 were also observed in livers with less vascular inflammation. Recent evidence has found that NRF2 deficiency may accelerate vascular senescence and calcification, calling for further investigation into NRF2 targeting in LT [301]. Given the ability of NRF2 to moderate both inflammatory and antioxidant pathways, it is likely that this finding, directly relating to hepatic inflow channels, occurred because of optimized tissue repair systems activated by higher NRF2 levels. Increased CD3⁺ cell infiltration was noted in livers with lower NRF2 expression indicating poor resolution of local inflammation. Activation of the NRF2 pathway has been shown to upregulate HO-1 expression which leads to the decreased expression of pro-inflammatory cytokines such as TGF- β 1, IL-1 β , IL-6 and TNF- α chemoattractants for leukocytes, monocytes, neutrophils, and other effector cells. Additionally HO-1 activation can limit NF- κ B and the overproduction of further cytokines and chemoattractants for leukocytes, monocytes, neutrophils, and other effector cells [302]. Surprisingly, while HO-1

was higher in discarded livers with higher NRF2 expression, this did not reach statistically significance, however, the trend indicated that this may have been achievable in a higher sample size. In addition, histological analysis showed that steatosis was significantly higher in low NRF2 livers and this finding parallels the widely reported interplay of the NRF2 axis in hepatosteatosis and the spectrum of NAFLD [303-305]. While the contribution of small droplet macrosteatosis to overall allograft function remains debatable [306-308], its presence can influence mitochondrial beta-oxidation and overall hepatotoxicity [309].

Discarded livers with higher levels of NRF2 demonstrated lower hepatic glycogen levels also corresponding to lower serum glucose. These associations are potentially explained by the role of NRF2 in insulin resistance. In a high fat diet murine model, Liu et al demonstrated hepatic insulin resistance through activation of the NF- κ B signaling pathway when NRF2 is deficient [310]. In the current set of experiments, the potential for glucotoxicity was evident in the low NRF2 livers and the strong PAS staining could suggest a worse glycogen profile. However, similar to inconsistent results on NRF2 and obesity, some studies appear to indicate increased insulin sensitivity and glucose homeostasis in NRF2 deficient systems [294, 311].

AMP – activated protein kinase (AMPK) is an intracellular energy sensor seen to play a central role in the regulation of glucose and lipid metabolism with studies suggesting it's activation in cellular energy depletion [312, 313]. Subsequent activation leads to the upregulation of catabolic pathways, most importantly glucose and fatty-acid oxidation, while reducing ATP consuming anabolic pathways such as cholesterol and triglycerol synthesis [314, 315]. Activation is facilitated by AMPK phosphorylation and the formation of pAMPK which contributes to energy dependent metabolic activities and the current study suggests a potential positive link between NRF2 and AMPK. The results could therefore suggest that livers with higher NRF2 are able to better regulate cellular energy sources and support continue metabolic activities in oxidative stress.

Keap-1 levels were lower in livers with high NRF2, however, this did not reach statistical significance. Initially, it was assumed that a high NRF2 environment was as a result of a low

Keap-1 environment but that was not the case. There could be several reasons for this observation. Firstly, in basal conditions, NRF2 and Keap-1 exists as a complex and therefore loss of one could automatically allude to loss of another. Secondly, due to the previously mentioned autoregulatory feedback loop of NRF2 whereby NRF2 activity also leads to Keap-1 transcription and production, the two factors may be balanced and thus an increase in NRF2 again does not negate the actions of Keap-1.

Higher hepatic NRF2 appeared associated with higher hepatic expression of pERK, an ER stress sensor that has been shown to play a role in cell proliferation and regeneration[316]. This observation in the current set of experiments could be viewed optimistically and indicate the ability of cells to regenerate and recover in a high NRF2 environment. This has been previously reported in mouse fibroblasts where NRF2-KO cells demonstrated increased cell death from ER stress [317]. In the setting of discarded allografts, the ability of cells to continue cellular metabolism functions while still proliferating and recuperating is a positive finding that would support their use in transplantation. However, like most transcription factors, overactivation of the ERK pathway could only be accepted with caution and evidence suggests that it can both inhibit and stimulate the proliferation of HepG2 cells in the context of hepatocellular carcinoma [318]. Thus, pERK may bidirectionally regulate cell proliferation depending on its intensity and a high NRF2 environment may still need to be controlled [316]. Cleaved-caspase-3 expression levels, although lower in livers with high NRF2 expression, did not reach significance. Previous studies on NASH have shown that increased hepatic inflammation was associated with increased CCP3 expression [319] and that this was associated with an increase in serum enzyme level. Thus, despite not showing a clear statistical difference, the clinical relevance remains important [320]. Similarly, LDH levels were comparable in both groups likely due to the fact that both groups of livers were equally hypoxic since LDH is essential for anaerobic respiration [321].

The expression of NRF2 was significantly increased in lean livers compared to steatotic livers and a number of mechanisms could be responsible for this observation. In a murine model, Yates and colleagues demonstrated that NRF2 activation suppresses fatty acid synthesis [322]. In another study, Tanaka and colleagues demonstrated that a 4 week high fat diet in mice reduce NRF2 mRNA and its target genes in wild type mice while also concluding that

NRF2 inhibits hepatic free fatty acid accumulation [323]. Moreover, Meakin and colleagues have shown that NRF2 deficient states lead to less fatty acid oxidation and higher lipogenesis [324] and therefore the findings from the current experiments support this literature. The novelty of the current work, however, is in the use of human allografts and discarded livers which suggest that to a certain extent steatotic livers with high NRF2 expression could still be useable. Additionally, the biochemical comparisons of lean and steatotic livers, as they relate to NRF2 expression, are worth considering. Lean livers with high expression levels of NRF2 had comparable serum transaminases to steatotic livers with similarly high expressions of NRF2. Steatotic livers with higher expression levels of NRF2 had slightly lower transaminases than lean livers with lower levels of NRF2. In both instances statistical significance was not reached. A move forward would be to speculate that NRF2 expression levels could be a determining factor in allograft functionality regardless of the degree of steatosis, however, additional samples from a variety of donors need to be studied to consider this true.

3.7 Implications

What is already known:

The NRF2 pathway has antioxidant, anti-inflammatory and cytoprotective cellular functions. Previous attempts to activate NRF2 have focused on stimulating these functions and many have been successful in small animal models. Previous studies have also indicated that NRF2 is reduced in ageing and hepatic steatosis and this may indicate the susceptibility of allografts from older donors or steatotic livers to IRI and early allograft dysfunction.

What is new:

There is wide variation in the expression of NRF2 in discarded human livers. Livers with greater expression levels of NRF2 demonstrated less injury and this was based on lower serum transaminases and histological inflammatory scores. Interestingly, most of the inflammation appeared to occur around the portal triad where we found increased inflammatory cell infiltration around the hepatic artery and portal vein. This indicated an impairment in hepatic inflow. Discarded livers with higher NRF2 expression demonstrated less toxic accumulations of hepatic glycogen. Encouragingly livers with higher expression of NRF2 also demonstrated a higher expression of pAMPK and pERK suggesting their ability to continue metabolic, energy dependent processes and cellular regeneration compared to livers with lower levels of NRF2 despite both groups being discarded in this setting. Additionally, hepatic steatosis had a negative impact on NRF2 expression and greater than 40% steatosis resulted in further NRF2 loss in most instances. Some steatotic livers with high NRF2 could remain functional and, clinically, demonstrated better biochemical profiles even when compared to lean livers with low NRF2.

Potential impact on future practice:

The results of the above experiments indicated the heterogeneity of discarded livers and suggest that some livers may still remain functional if the NRF2 axis is intact. In most instances, a higher NRF2 environment resulted in more favourable clinical parameters. NRF2 could be a marker when determining the quality of discarded or marginal livers for transplantation. How high NRF2 livers respond to IRI in a transplant setting is the next logical step after the above experiments.

Chapter 4

NRF2 as a biomarker during normothermic machine perfusion

4.1 Introduction

Not all donated livers offered are suitable for transplantation. Between 2014 and 2015, of the 1282 donors identified in the UK, only 72% of livers were deemed suitable for retrieval and only 63% were subsequently transplanted [74]. Similar data from the USA indicates that less than 80% of livers are eventually transplanted [37]. To address the ever expanding patient population, there has been recent interest in using marginal or extended criteria livers, however, this is done with caution. In most instances, marginal livers are accepted or declined based on subjective surgeon assessments combined with liver biopsy results and standard donor information [325]. Additionally, the donor risk index (DRI) was devised in 2006 and is currently used in the US as a marker of allograft quality and can help make decisions about organ discard to specifically predict the risk of post-transplant graft failure [326]. Seven donor factors and two procurement factors are combined into the DRI model: donor age, race, height, death from a cerebrovascular accident, donation after cardiac death (DCD), cause of death classified as “other”, split grafts, cold ischaemia time and the location of the organs based on the donation service area [327]. In recent years, there has been evidence criticising the model in its inability to accurately predict allograft failure and more modern predictive tools have been devised [325] but all remain limited in their translation to clinical practice.

Static cold storage has been the standard method of organ preservation after procurement [62]. Using this method, organs are flushed and cooled with one of two preservation fluids University of Wisconsin (UW) solution or histidine–tryptophan–ketoglutarate (HTK) solution. The organs are then placed in plastic bags that are filled with preservation fluid within an ice box and transported to the donor. The induced hypothermia slows down the metabolic processes by 12 fold but does not prevent cellular dysfunction [328]. This ischaemic phase triggers the complex immunological cascade and the release of pro-inflammatory mediators that are subsequently worsened during the “reflow paradox” stage of organ reperfusion upon implantation of the liver [329]. As such, ischaemic injury that occurs during cold storage and limits in preservation time make this method of preservation less than ideal. In recent years normothermic machine perfusion (NMP) has gained popularity. The main advantage of this technology over hypothermic machine archetypes is the ability to preserve metabolic activity preventing further graft damage while also allowing for

objective biochemical assessments. In a landmark study by Ravikumar et al in a phase 1 trial, the authors compared NMP and cold stored livers, initially reporting that NMP livers had significantly lower transaminases 7 days post op when compared to the cold group [330]. Following this innovation, Mergental and colleagues from Birmingham were then among the first to publish their results on recipients of NMP treated discarded livers [74]. Four of the five livers were from DCDs and all grafts functioned immediately after transplant. Viability assessments were based on perfusate lactate clearance, bile production and stable blood flow rates. Moreover, a multicenter European RCT with 220 liver transplantations comparing static cold storage to NMP found the latter to be associated with 50% lower levels of allograft injury and, most importantly, a 50% lower rate of organ discard [331]. After this achievement, Mergental et al once more published their work and the results of the Viability Testing and Transplantation of Marginal Livers (VITTAL) trial assessing 31 livers using NMP and a refined set of viability criteria [332]. Remarkably, satisfactory viability testing was achieved in 71% of discarded livers that were eventually transplanted.

Naturally, not all discarded livers will likely be rescued but identifying which allografts to salvage is a worthy cause that could ultimately reduce wait list mortality. There is a constant need to improve methods by which high risk livers are assessed and hepatic biomarkers may offer information on how a liver will function on the machine.

Hypothesis:

The NRF2 axis influences discarded allograft function and can be considered a biomarker for marginal liver selection.

Overall aim:

To study the role of hepatic NRF2 in allograft function, injury and repair systems.

4.2 Specific aims

The aims of this chapter include:

- Investigating NRF2 changes during normothermic machine perfusion (NMP).
- Investigating the functional and molecular impact of NRF2 on liver allografts during NMP.
- Assess NRF2 modulations in fatty livers during normothermic machine perfusion.

4.3 Results

4.3.1 Hepatic NRF2 responses to normothermic machine perfusion

Livers (n=8) were selected at random and NRF2 protein was quantified by Western blot. Livers were ranked and divided into two groups based on high and low NRF2 expression levels. Donor characteristics are detailed in Table 4.1.

Table 4. 1 Donor characteristics of livers undergoing normothermic machine perfusion[282].

Variables	High NRF2 expression	Low NRF2 expression
N	4	4
Donor age, median (range)	51 (49 – 54)	46 (34 – 57)
Gender M:F	3:1	3:1
BMI kg/m ² , mean (SD)	25.1 (7.5)	32.4 (2.2)
Donor cause of death		
Anoxia	2	3
CVA	1	1
Trauma	1	-
Cause for organ discard		
Exceeded DCD time	2	2
Donor age	1	1
Steatosis	1	1
Median WIT (minutes)	56	26
Lean : Steatosis	3:1	1:3

BMI; body mass index, CVA; cerebrovascular accident, DCD; donation after cardiac death, WIT; warm ischaemia time.

Hepatic NRF2 responses were again investigated after 6 hours of normothermic machine perfusion. Baseline NRF2 expression at 0 hours did not differ significantly (Figure 4.1a). Livers from the high NRF2 group demonstrated a 36.5%-fold increase in NRF2 following NMP, while livers from the low NRF2 group saw a further 19.5% decrease in expression levels (Figure 4.1b).

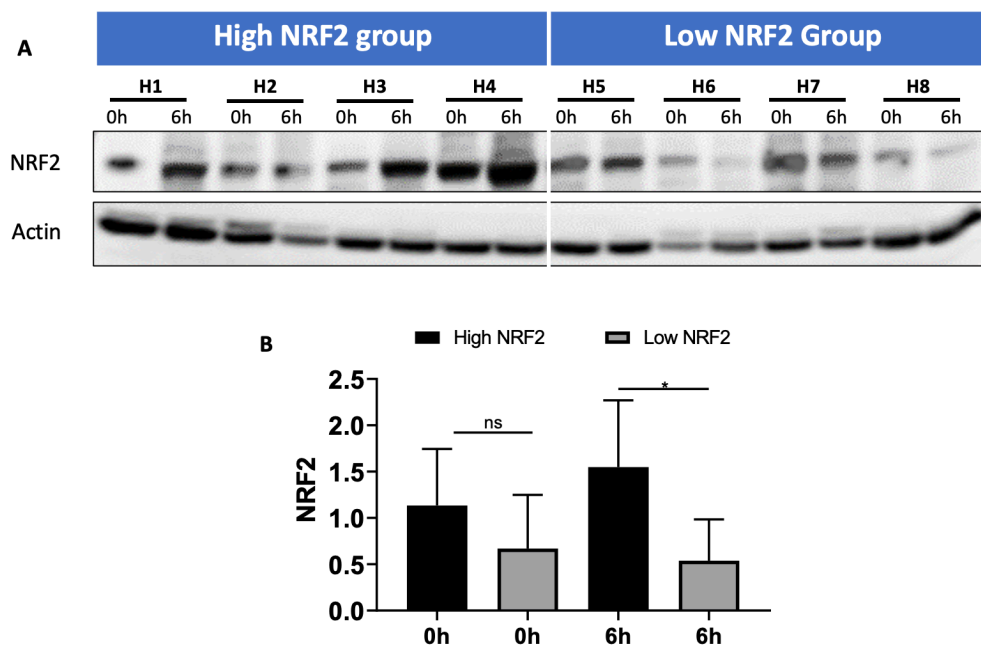


Figure 4. 1 Changes to NRF2 expression following ex vivo normothermic machine perfusion (NMP)[282]. Expression of NRF2 was determined by Western blotting at the 0 and 6-hour time-point in high and low NRF2 group discarded human livers. a) Higher NRF2 expression is maintained in high NRF2 group livers after NMP. b) Densitometric analysis of Western blotting for NRF2 expression demonstrated significantly higher levels of NRF2 expression after NMP. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant by t-test.

Immunofluorescent staining of livers pre and post-NMP was also performed to assess for changes in NRF2 translocation and to also determine the location of NRF2 within liver cells. First, baseline nuclear NRF2 intensity appeared higher at 0 hours before NMP in livers with higher expression levels of NRF2 and this increased even further after 6 hours of NMP (Figure 4.2)

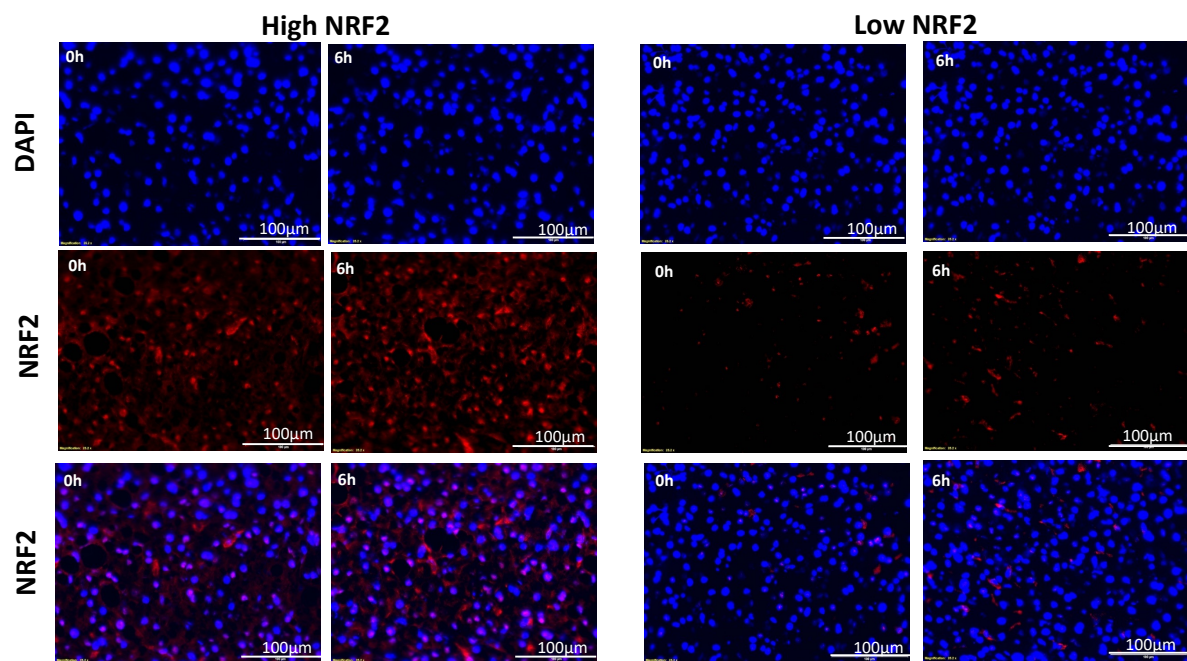


Figure 4. 2 Representative micrographs of immunofluorescence staining for NRF2 (red) and nuclei (DAPI; blue) in high and low NRF2 group discarded human livers following ex vivo normothermic machine perfusion (NMP)[282]. Increased NRF2 expression was observed in livers with higher NRF2 levels which was evident in both nuclear and cytoplasmic content on immunofluorescent staining. Livers with already lower levels of NRF2 did not increase NRF2 significantly.

Using a double IF staining method, NRF2 content was then specifically assessed in hepatocytes (Figure 4.3), cholangiocytes (Figure 4.4) and macrophages (Figure 4.5). Positive staining confirmed that NRF2 was present in all 3 cell types before and after NMP.

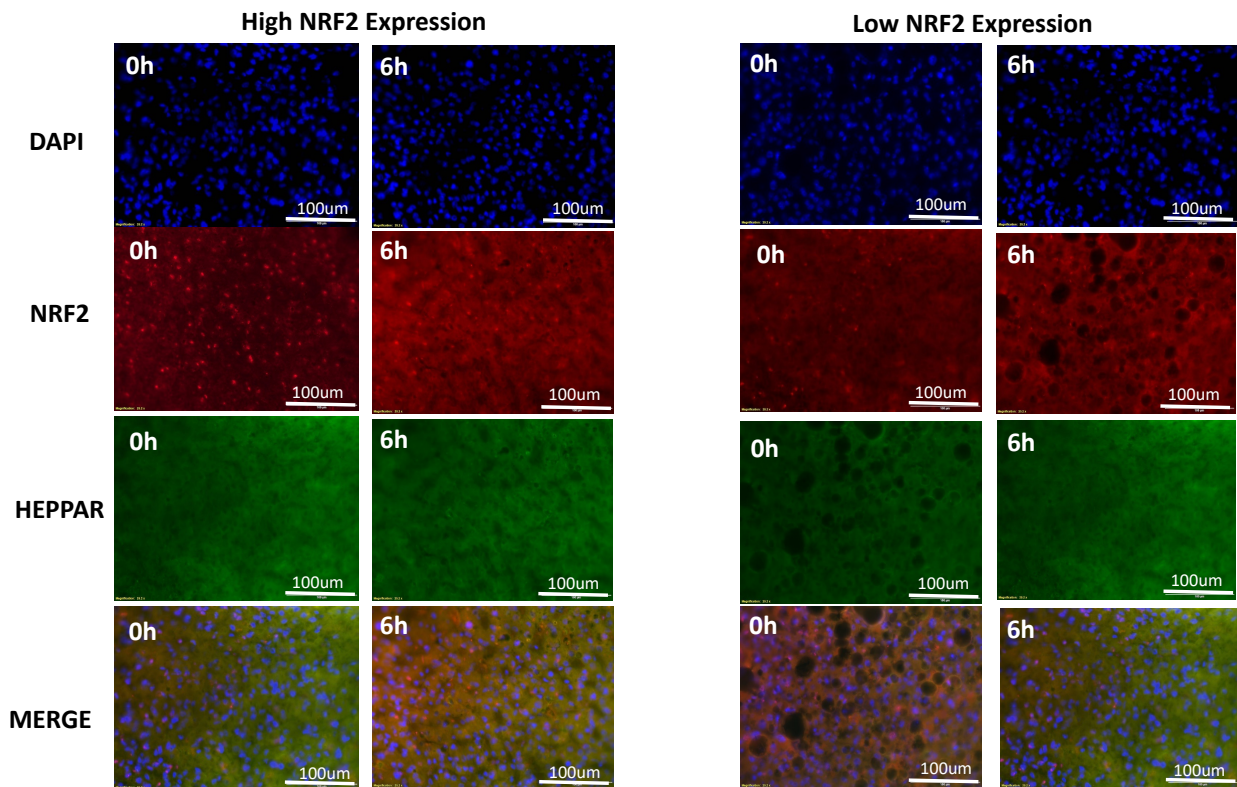


Figure 4. 3 NRF2 expression in hepatocytes in high and low NRF2 livers[282].

Immunofluorescent staining of high and low NRF2 group human discard livers for NRF2 (red), hepatocytes (HEPPAR, green) and nuclei (DAPI, blue). NRF2 immunoreactivity was observed at variable levels in hepatocytes in both groups.

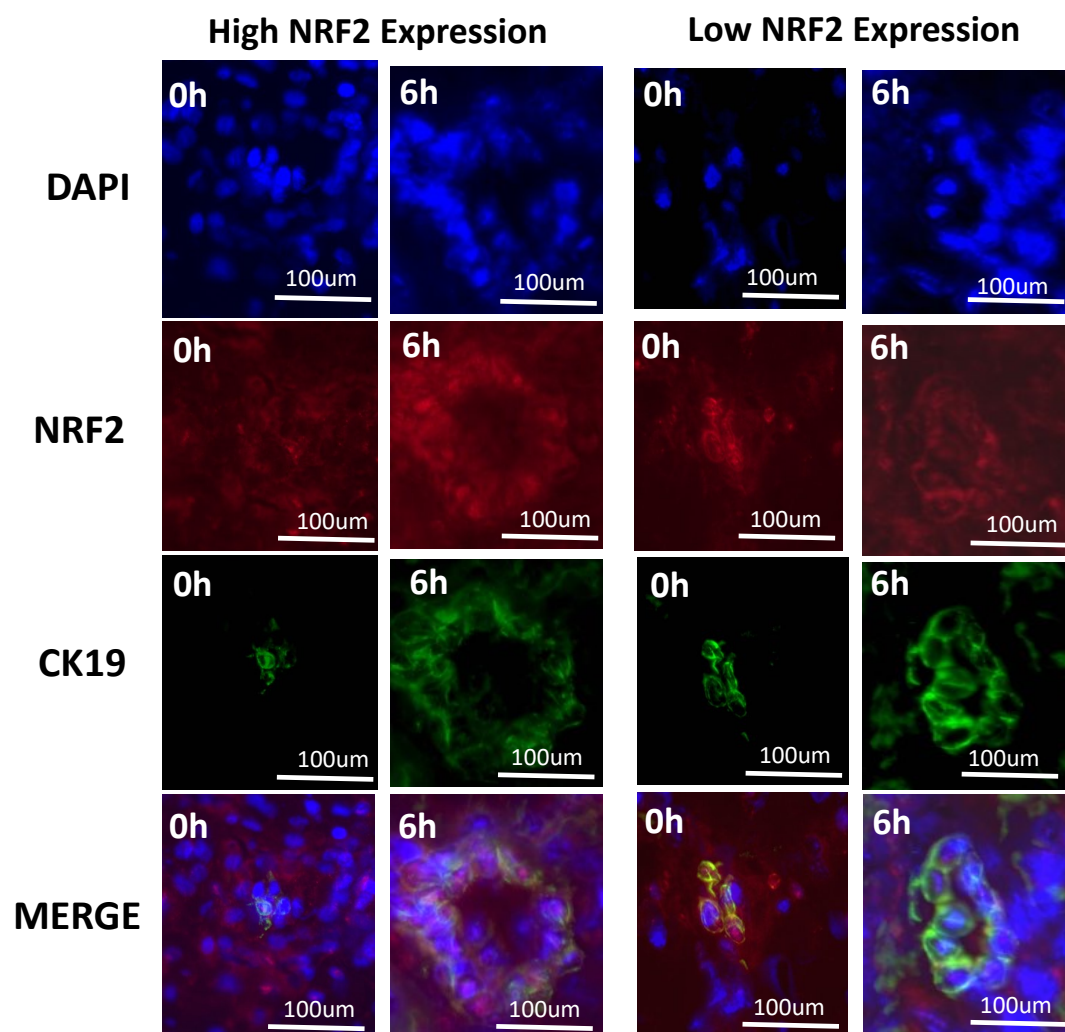


Figure 4. 4 NRF2 expression in cholangiocytes in high and low NRF2 livers[282].

Immunofluorescent staining of high and low NRF2 group human discard livers for NRF2 (red), cholangiocytes (CK19, green) and nuclei (DAPI, blue). NRF2 immunoreactivity was evident in cholangiocytes in both high and low NRF2 groups.

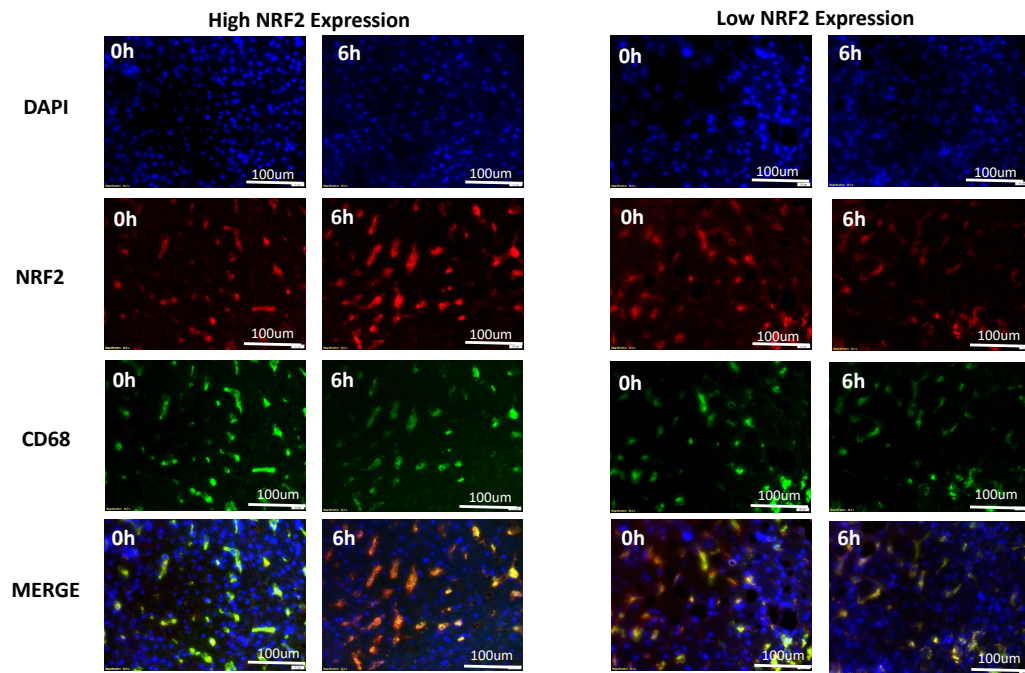


Figure 4. 5 NRF2 expression in hepatic macrophages in high and low NRF2 livers[282]. Immunofluorescent staining of high and low NRF2 group human discard livers for NRF2 (red), hepatic macrophages (CD68, green) and nuclei (DAPI, blue). NRF2 expression was observed in hepatic macrophages in both high and low NRF2 groups.

4.3.2 Biochemical analyses in high and low NRF2 livers after NMP

Following 6 hours of NMP, mean ALT was significantly lower in livers with high NRF2 levels ($5,919.4 \pm 4,423.6$ iU/L) when compared to livers with lower NRF2 levels ($12,001.5 \pm 2,961.5$ iU/L; $p = 0.048$). A similar trend was observed for post-NMP AST ($7,818.0 \pm 4,743.0$ iU/L vs $33,405.0 \pm 16,117.1$ iU/L, $p = 0.029$) and post-NMP alkaline phosphatase (174.0 ± 82.0 iU/L vs 428.0 ± 25.4 iU/L, $p = 0.006$) which were both significantly lower in livers with higher NRF2 expression levels (Figure 4.6). Increased lactate clearance following NMP was also achieved in livers with high levels of NRF2 (0 hours = 1.6 mmol/L vs 2 hours = 0.5 mmol/L) when compared to livers with lower NRF2 (0 hours = 3.7 mmol/L vs 2 hours = 3.0 mmol/L). These livers showed acceptable glucose levels at the end of perfusion (265 mg/dL vs 92 mg/dL, $p = 0.028$) and hepatic glycogen restoration, which increased, although not significantly. Additionally, serum bilirubin and albumin did not change significantly after 6 hours of machine perfusion with high and low NRF2 livers were compared (Figure 4.7).

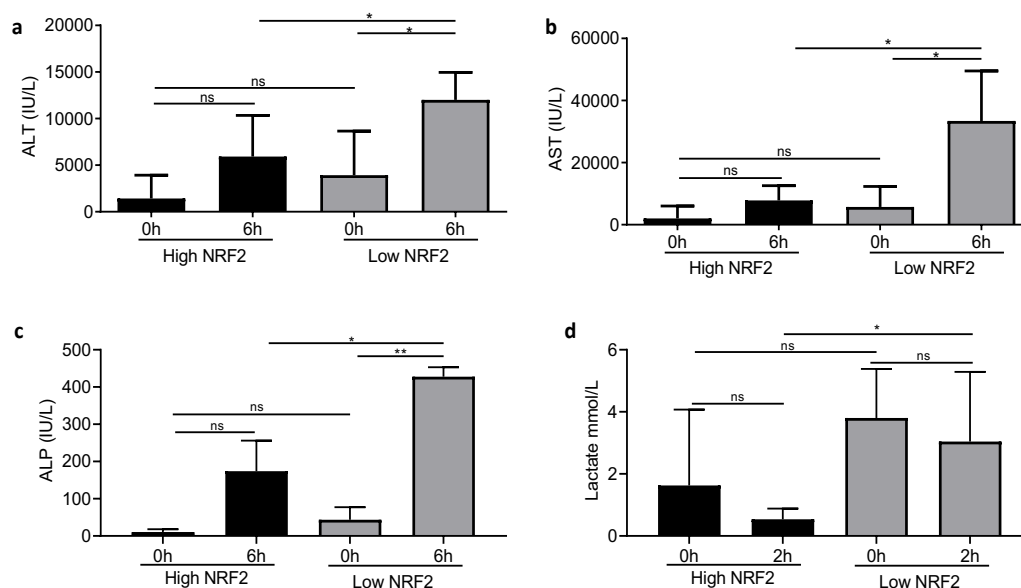


Figure 4. 6 High NRF2 livers have improved liver function after NMP[282]. Biochemical assessment of perfusate from livers before and after normothermic machine perfusion (NMP). High NRF2 group livers had lower a) alanine aminotransferase (ALT), b) aspartate aminotransferase (AST), c) alkaline phosphatase following 6 hours or NMP d) and improved lactate clearance at 2 hours post-NMP. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

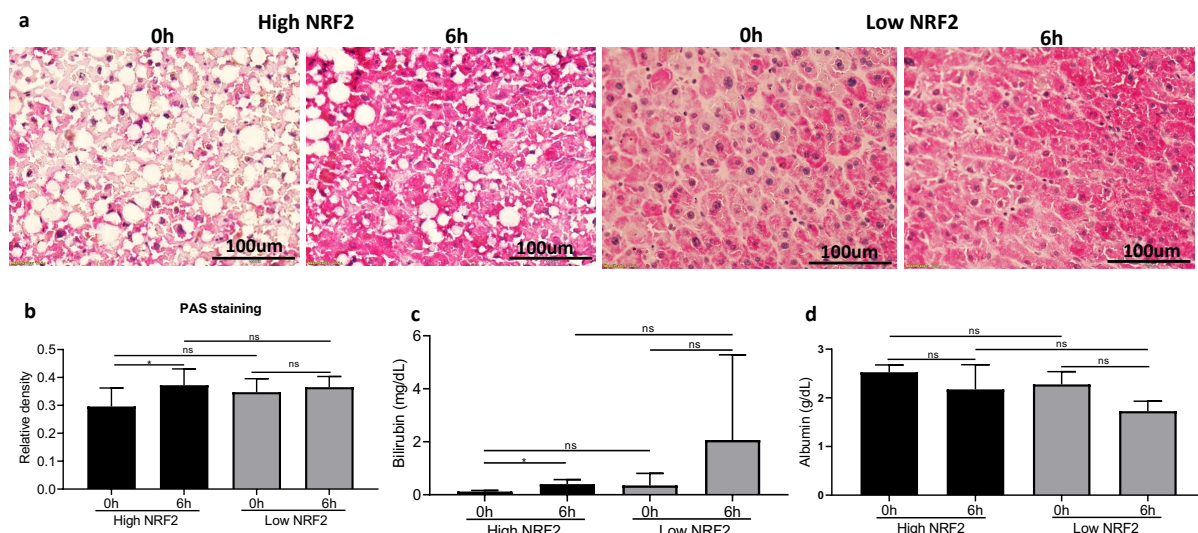


Figure 4. 7 Periodic acid Schiff (PAS) staining, bilirubin and albumin levels in high and low NRF2 livers after NMP. **A)** Objectively, hepatic glycogen content appeared to increase dramatically in both livers with high and low NRF2 expression, although **B)** this did not reach statistical significance. **C)** There was a significant increase in bilirubin levels in the high NRF2 expression liver group after 6 hours of NMP and similar increase in low NRF2 expression levels although this did not differ significantly from basal levels. There were not significant differences in serum bilirubin between high NRF2 and low NRF2 livers after 6 hours of NMP. **D)** In both groups, serum albumin appeared to decrease post perfusion, however this did not change significantly from basal levels at 0 hours or between both groups after 6 hours of NMP. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant by t-test.

4.3.3 Parenchymal inflammation in high and low NRF2 livers after NMP

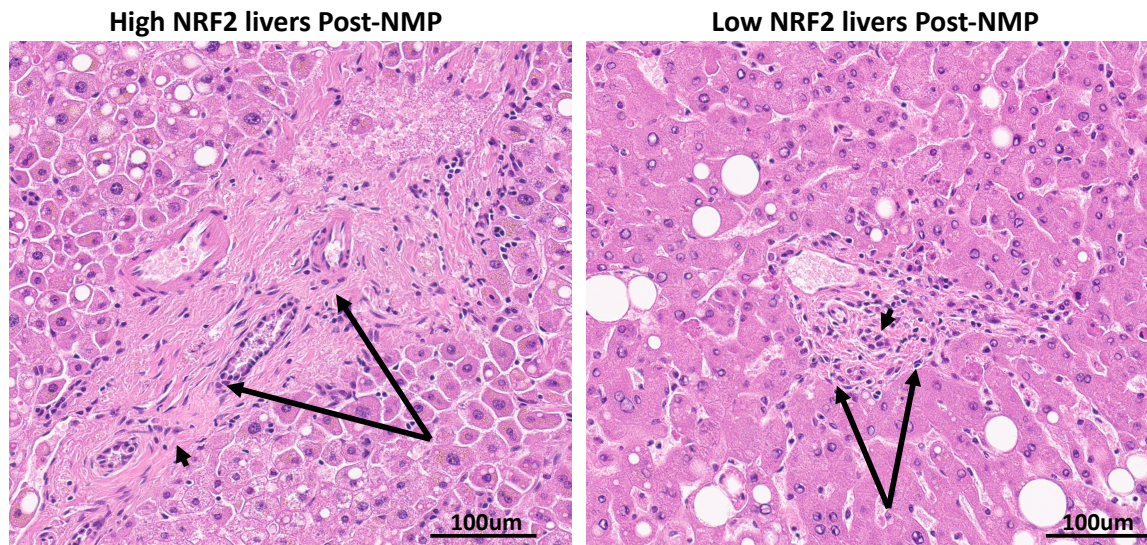
The extent of inflammatory injury was once more assessed histologically on discarded liver biopsies that were taken before and after 6 hours of NMP. Inflammatory scores were determined on H&E staining by a pathologist who was blinded to the NRF2 categories and the timepoints of each sample. Livers with high expression levels of NRF2 scored better in all Banff and Suzuki Scoring criteria. Livers with high NRF2 expression levels maintained lower hepatic artery inflammatory scores ($p = 0.02$) after NMP as detailed on Table 4.2.

Table 4. 2 Histological variables related to allograft NRF2 expression following 6 hours of normothermic machine perfusion[282].

	High NRF2 Expression	Low NRF2 Expression	P value
Necrosis	0	0.3	0.37
Bile duct inflammation	1.3	2	0.12
Vascular inflammation			
Hepatic artery	0	1.3	0.02
Hepatic vein	0	1	-
Portal vein	0	1	0.16
Vacuolization	1	0	0.37
Peri-portal inflammation	1	1.3	0.37

Livers with lower expression levels of NRF2 achieved higher inflammatory score categories (scores 2 – 3) after 6 hours of NMP (Figure 4.8). Although necrosis remained higher in livers with low expression levels of NRF2, this did not achieve statistical significance.

a



b

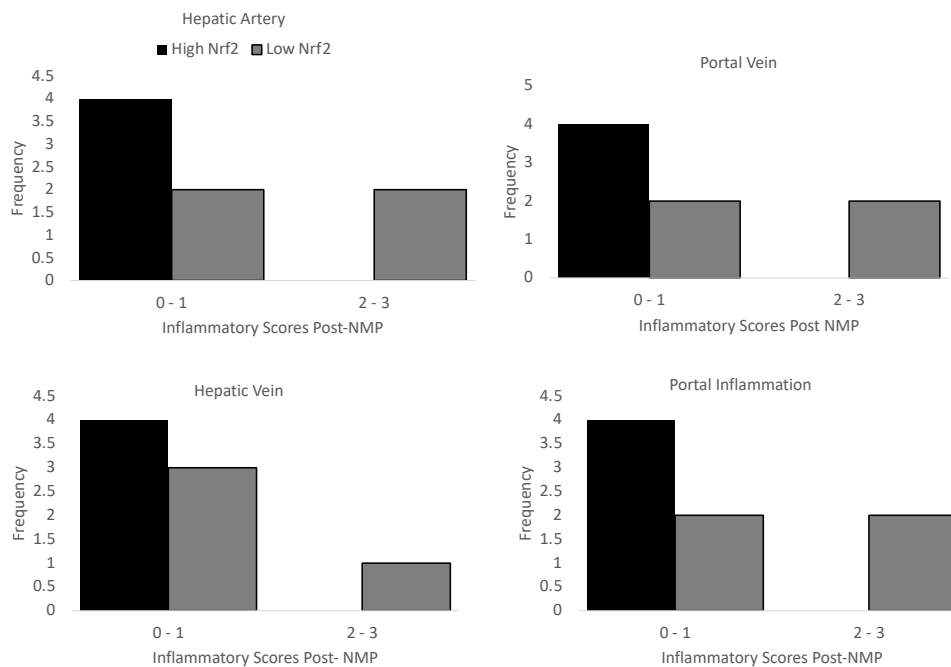


Figure 4. 8 Hepatic inflammation on hematoxylin and eosin staining of discarded human livers after 6 hours normothermic machine perfusion (NMP)[282]. **a)** Livers with higher NRF2 expression demonstrated increased inflammatory infiltration around the porta triad (arrows). High NRF2 livers showed lower hepatic artery inflammation (arrow heads). High NRF2 livers observed lower vascular inflammatory scores (0-1). **b)** Histograms representing histological inflammatory scores in discarded livers after 6 hours of normothermic machine perfusion. High NRF2 livers showed lower hepatic artery inflammation and demonstrate the absence of any high NRF2 liver with vascular inflammatory scores greater than 1 after 6 hours of machine perfusion.

To specifically determine the inflammatory cell type in portal inflammation after 6 hours of NMP, IF staining was again performed specifically targeting CD3+ cell phenotypes (Figure 4.3.3). Quantitation showed that CD3+ cells were significantly lower in livers with high NRF2 expression levels who underwent 6 hours of NMP when compared to livers with low NRF2 levels ($p=0.034$) at the same timepoint.

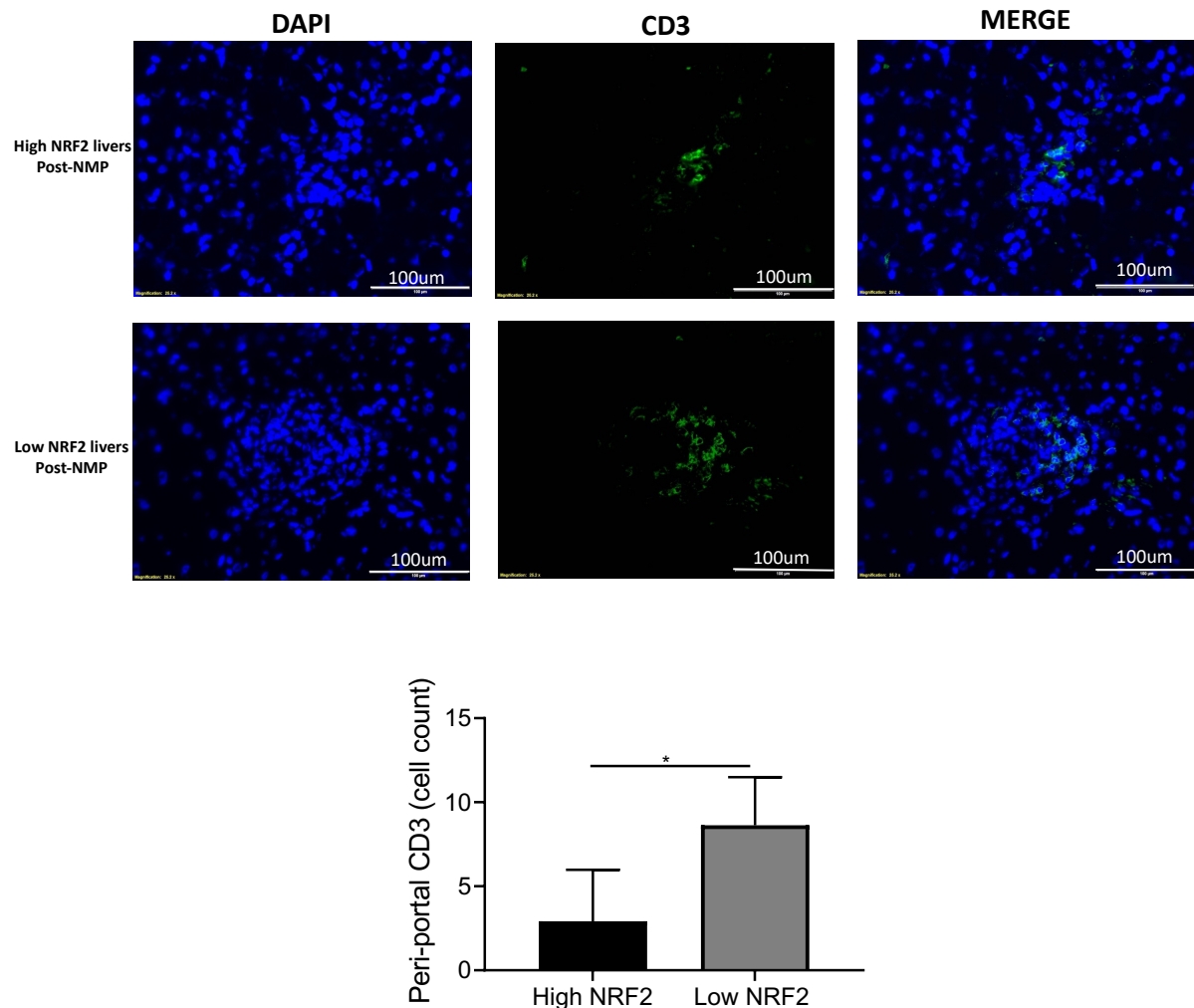


Figure 4. 9 Immunofluorescent staining for T-cells (CD3, green) and nuclei (DAPI, blue) in discarded donor livers after normothermic machine perfusion (NMP)[282]. Quantitation revealed reduced portal T cell infiltration in high NRF2 livers after NMP. Data are represented as mean $\pm$ SD. *, $p < 0.05$ by t-test.

4.3.4 Molecular changes during normothermic machine perfusion

To evaluate the molecular impact of NRF2 on livers during NMP, previously assessed molecular markers were once again evaluated for changes after NMP; pAMPK, HO-1, Keap-1, pERK and CCP-3.

Pre- and post-NMP liver samples were assessed for AMPK phosphorylation (Figure 4.10). Similar to the first batch of liver samples, baseline pAMPK at 0 hours was significantly higher in the high NRF2 group (2.03 ± 0.91 versus 0.9 ± 0.5 , $p = 0.036$). Hepatic pAMPK levels decreased in both groups following 6 hours of NMP and this was more significant in the high NRF2 group (0 hours = 2.02 ± 0.91 , 6 hours = 0.88 ± 0.05 , $p = 0.0346$). Interestingly, the low NRF2 group saw a similar decrease in pAMPK levels (0 hours = 0.9 ± 0.5 , 6 hours = 0.4 ± 0.268 , $p = 0.06$) but this change did not reach statistical significance. Total AMPK levels increased in both the high and low NRF2 group following 6 hours of perfusion and was significantly higher in the low NRF2 group after NMP (3.73 ± 0.32 versus 0.83 ± 0.52 , $p = 0.37$).

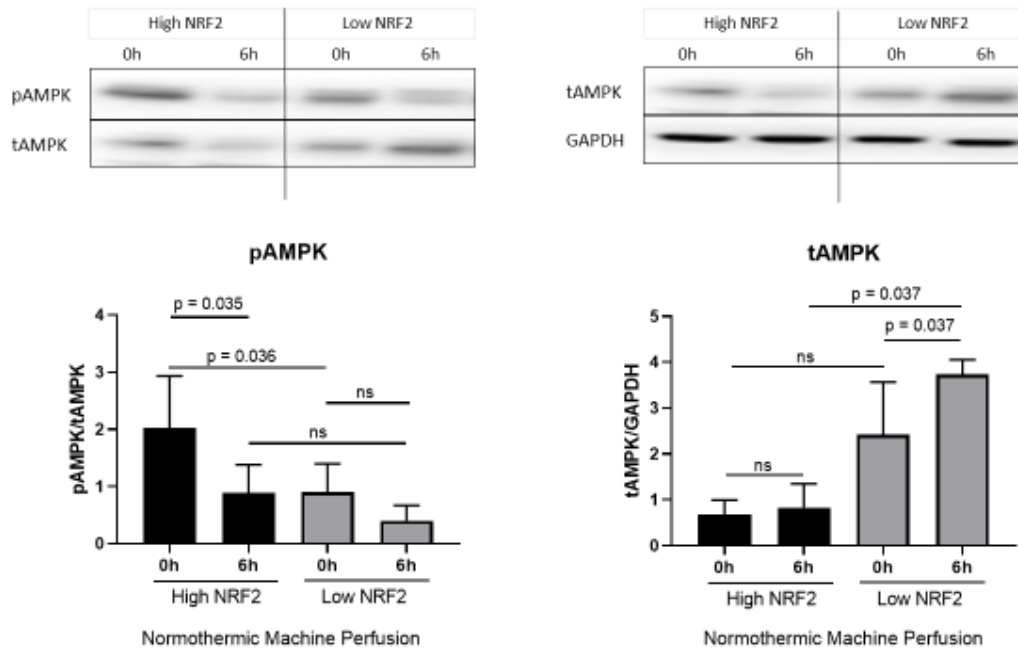


Figure 4. 10 Changes to AMPK expression in relation to NRF2 expression following 6 hours of NMP. Livers with high NRF2 expression demonstrated significantly decreased pAMPK expression after 6 hours of machine perfusion compared to baseline levels at 6 hours. Although low NRF2 livers also decreased pAMPK expression after NMP, both high and low NRF2 expression livers had comparable levels of pAMPK after NMP. There was no significant changes to tAMPK in livers with high NRF2. Low NRF2 livers demonstrated significantly higher levels of tAMPK from 0 to 6 hours after NMP and expression at 6 hours was significantly higher than expression at 6 hours in the high NRF2 group. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

To evaluate the downstream antioxidant pathway, hepatic HO-1 expression levels were examined on western blotting (Figure 4.11). Interestingly, although both the high NRF2 (0 hours = 5.81 ± 6.77 , 6 hours = 2.39 ± 1.57) and the low NRF2 (0 hours 0.69 ± 0.47 , 6 hours 0.66 ± 0.4) group demonstrated a decrease in levels after NMP, livers with high NRF2 expression still had significantly higher HO-1 at 6 hours compared to the low group at the same time point.

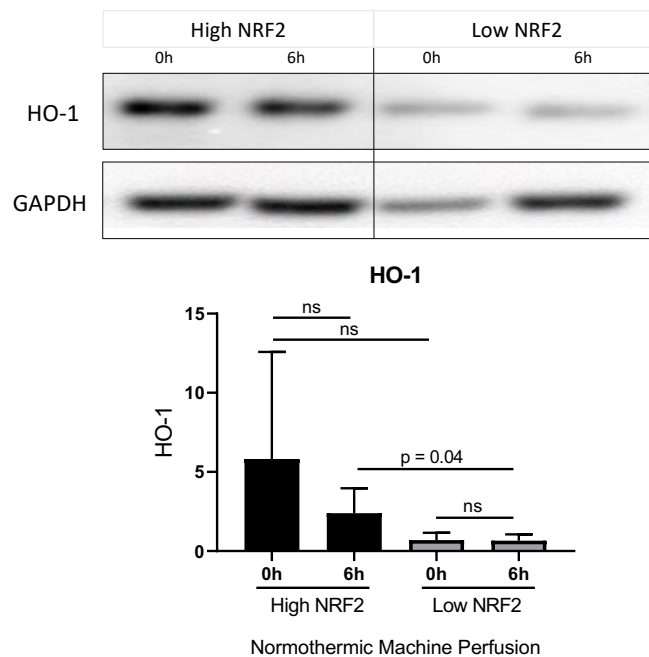


Figure 4. 11 Changes to HO-1 expression in relation to hepatic NRF2 after NMP. In both liver groups (high and low NRF2 expression), HO-1 levels appeared to decrease after NMP. Livers with high NRF2 expression still maintained higher HO-1 expression that livers with low NRF2 levels by the end of NMP. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

Hepatic expression of Keap 1 appeared similar at 0 hrs prior to NMP in both high NRF2 and low NRF2 groups (1.18 ± 0.21 versus 2.29 ± 1.17 , $p = 0.56$). Both groups, however, responded differently to NMP. High NRF2 livers showed a decrease in Keap 1 levels following 6 hours of machine perfusion (0 hours = 1.18 ± 0.21 versus 6 hours = 0.71 ± 0.196 , $p = 0.02$). When livers with lower levels of hepatic NRF2 expression were exposed to NMP, Keap 1 expression remained unchanged (0 hours = 2.3 ± 1.17 versus 6 hours = 2.22 ± 3.7 , $p = 0.49$) (Figure 4.12).

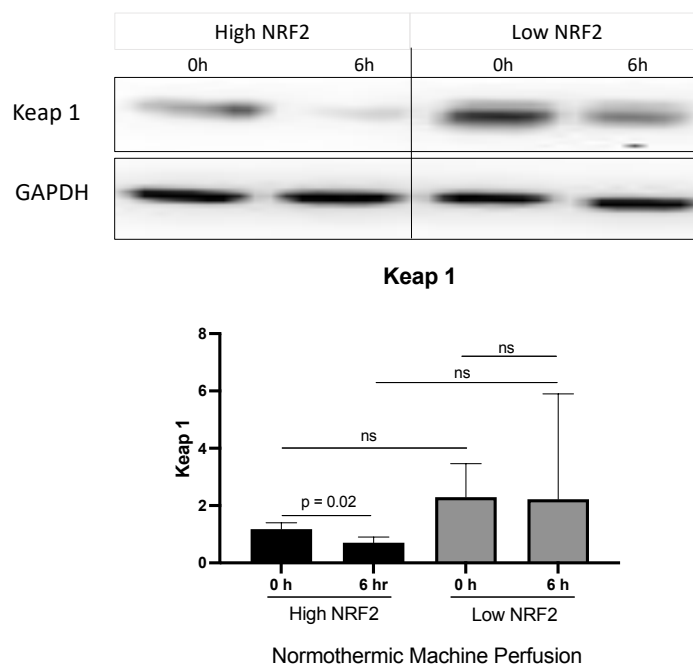


Figure 4. 12 Changes to Keap 1 expression in relation to hepatic NRF2 after NMP. Livers with high NRF2 decreased Keap 1 levels after 6 hours of NMP, however, both groups had comparable levels after 6 hours of machine perfusion. Data are represented as mean $\pm$ SD.

*, $p < 0.05$; NS = not statistically significant.

Prior to NMP, the high NRF2 group had significantly higher levels of ERK phosphorylation at the 0 hour time point (high NRF2 group 0 hours = 0.77 ± 0.63 versus low NRF2 group 0 hours = 1.62 ± 1.11 , $p = 0.048$). Both groups increased ERK phosphorylation after 6 hours of NMP, however, this was only significant in the low NRF2 group (high NRF2 group 0 hours = 0.77 ± 0.63 , 6 hours = 1.62 ± 1.11 , $p = 0.12$; low NRF2 group 0 hours = 0.14 ± 0.08 , 6 hours = 0.64 ± 0.47 , $p = 0.04$) and levels at 6 hours did not differ significantly between the 2 groups ($p = 0.08$) (Figure 4.13).

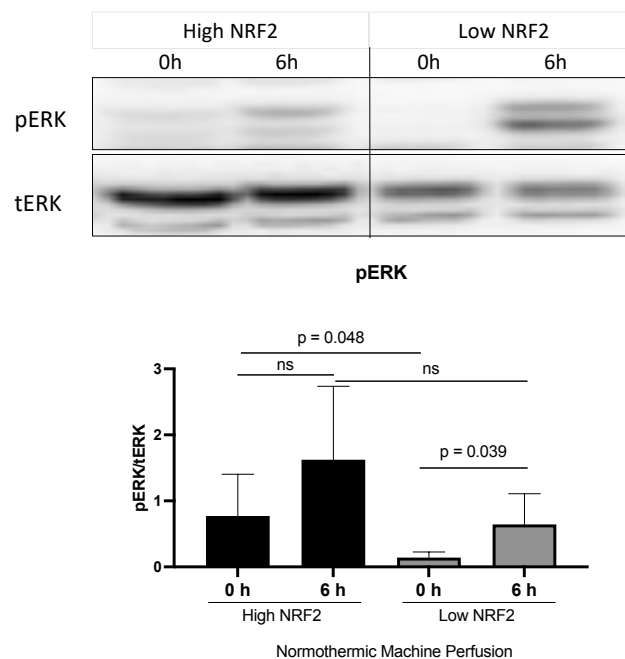


Figure 4. 13 Changes to pERK expression in relation to NRF2 after NMP. Both groups demonstrated an increase in pERK expression after 6 hours of NMP but the changes were only significant in the low NRF2 expression liver group. By the end of NMP, pERK expression was comparable between high NRF2 and low NRF2 expression livers. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

Caspase-3 expression decreased in both the high NRF2 (0 hours = 2.16 ± 2.36 , 6 hours = 1.47 ± 0.39 , $p = 0.29$) and low NRF2 group livers (0 hours = 3.82 ± 2.3 , 6 hours = 1.52 ± 1.8 , $p = 0.167$) after 6 hours of perfusion, however, significance was not reached in any case. Hepatic CCP-3 expression did not differ significantly in both groups at 0 hours ($p = 0.053$) or 6 hours ($p = 0.96$) (Figure 4.14).

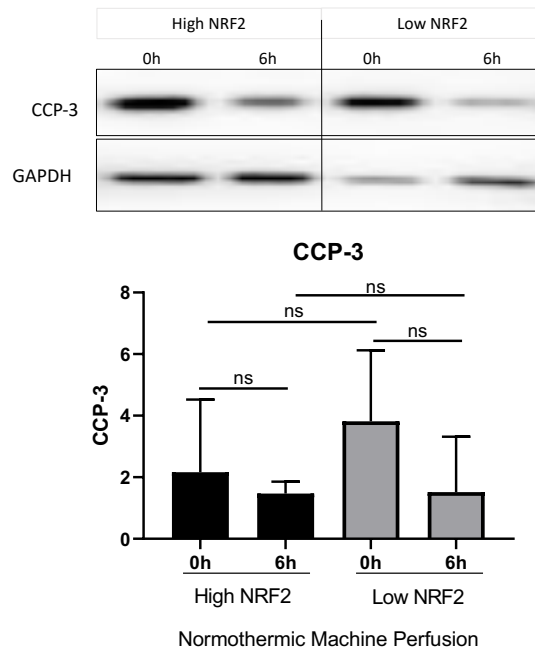


Figure 4. 14 Changes to CCP-3 expression in relation to NRF2 after NMP. In both groups of livers, CCP-3 expression appeared to decrease, however this did not reach statistical significance. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

4.4 NRF2, steatosis and responses to Normothermic Machine Perfusion

Livers exposed to NMP were analyzed based on lean and steatotic status and NRF2 responses were studied (Figure 4.15). After 6 hours of machine perfusion, lean livers demonstrated a significant increase in hepatic NRF2 expression on densitometric analysis (mean 0 hrs 1.44 ± 1.08 to 6 hrs 2.10 ± 1.18 ; $p = 0.0002$). In contrast, steatotic allografts showed a significant decrease in the level of NRF2 expression when the 0 hr and 6 hr timepoints were compared (mean 0 hrs 1.26 ± 0.84 to 6 hrs 0.88 ± 0.69 ; $p = 0.007$). Lean livers expressed higher levels of hepatic NRF2 by the end of the 6 hours perfusion time when compared to steatotic allografts (mean steatosis $37\% \pm 43\%$), which had much lower levels of NRF2 ($p = 0.028$).

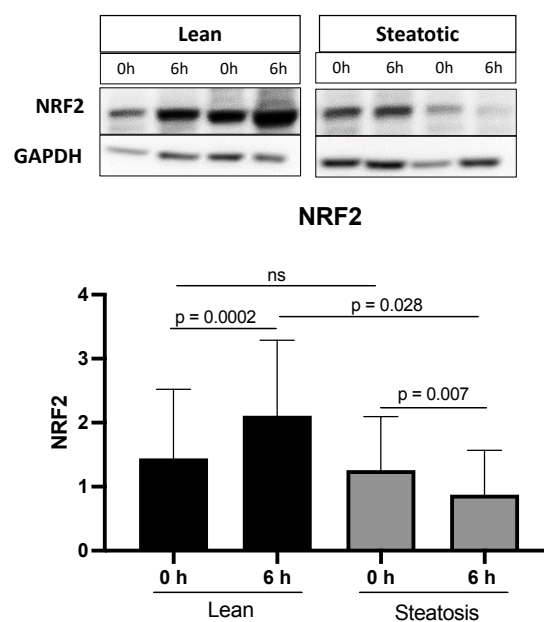


Figure 4. 15 NRF2 changes in lean and steatotic livers exposed to 6 hours of NMP. Lean livers appeared to have a positive response to NRF2 after 6 hours of machine perfusion compared to steatotic livers which either remained static or saw a decrease in NRF2 levels after NMP.

As previously, IF staining was used to assess changes to the nuclear translocation of NRF2 which was determining by the degree of nuclear quantitation. Increased nuclear NRF2 intensity was confirmed in lean livers following NMP (Figure 5.16).

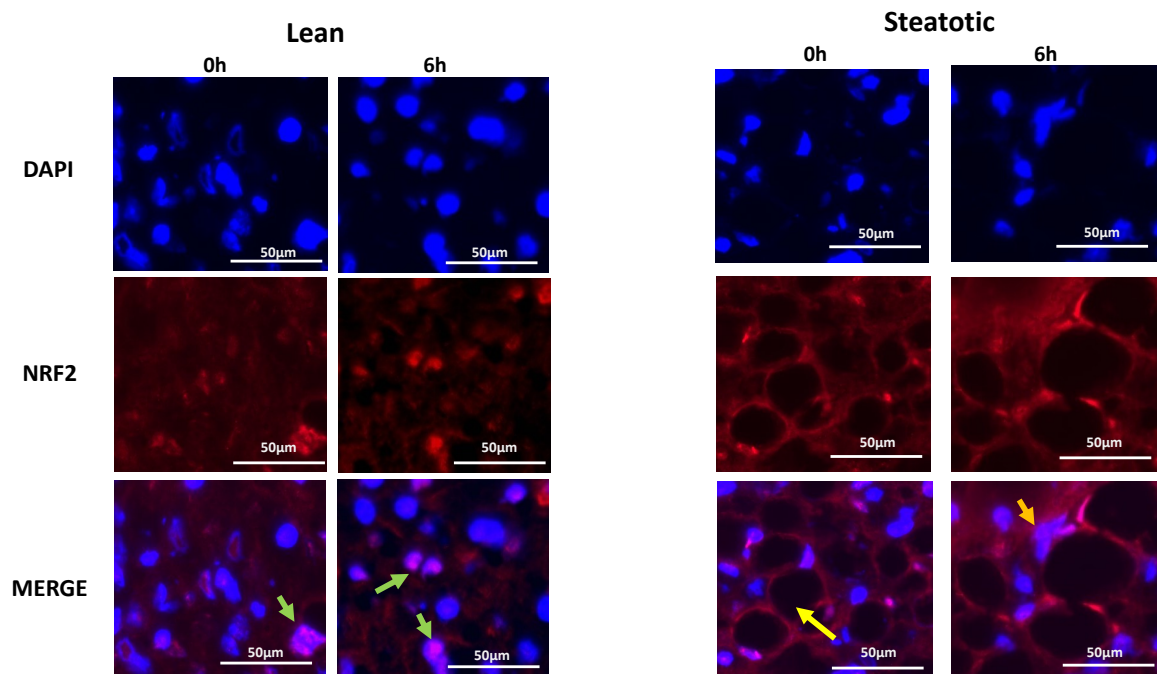


Figure 4. 16 Immunofluorescent staining for NRF2 (red) and nuclei (DAPI, blue) in discarded donor livers after normothermic machine perfusion (NMP). Lean livers demonstrate a high intensity of red nuclear staining indicating NRF2 translocation (green arrows). Steatotic cells (yellow arrows showing fat droplets) appear to show lower nuclear intensity suggesting less NRF2 translocation after NMP.

4.5 Discussion

Normothermic machine perfusion combats the previously described limitations of static cold storage and maintaining the liver at the body's natural temperature has the ability to provide a homeostatic environment in which adequate cellular metabolism can occur [73]. A number of important observations can be made from the above experiments. When livers with a high NRF2 expression are exposed to 6 hours of NMP, a positive response i.e. an increase in hepatic NRF2 expression is shown in contrast to livers with low expression levels of NMP which appear to decrease NRF2 levels. Less inflammatory injury is observed in livers with high NRF2 levels after NMP in terms of liver enzymes, lactate clearance and, interestingly, hepatic artery inflammation scores. We postulated that since the previous experiments demonstrated a higher expression level of NRF2 in lean livers over steatotic samples, these differences would persist in the setting of NMP. Interestingly, steatotic livers are shown to significantly lose hepatic NRF2 expression levels while lean livers experience a significant increase.

NRF2 has not been studied in the setting of NMP and discarded livers prior to these experiments, owing to the relative novelty of NMP. Xue and colleagues have previously attempted to study the NRF2 axis in the context of DCD livers exposed to hypothermic machine perfusion (HMP) [333]. In a murine model, the authors compared 3 hours of HMP to cold storage and report activation of the NRF2-ARE signaling pathway in livers exposed to machine perfusion which also resulted in lower liver enzymes and apoptosis. In the current set of experiments, livers that increased NRF2 expression showed significantly lower transaminases than livers with lower NRF2 expression levels which appeared to demonstrate a greater degree of injury. The data, therefore, may suggest that NRF2 may serve a role in the protective effects of NMP particularly in the antioxidant cellular defense mechanisms. Additionally, Xue et al also demonstrated a significant decrease in HO-1 expression in DCD livers after HMP, mirroring the findings from the above experiments where HO-1 appeared to decrease after 6 hours of NMP in both NRF2 groups. One possible explanation for this finding is that activation of the HO-1 gene has been shown to be regulated by the nuclear factor- κ B binding site, a heat-shock element and hypoxic response elements and not just components of the NRF2 axis [333-336]. This could also explain why HO-1 was still significantly higher in high NRF2 livers after 6 hours of NMP when compared

to low NRF2 livers at the same timepoint. An alternative explanation is that HO-1 has a relatively short half-life of 2 hours [337] and it is possible that it may have been expressed in higher quantities during the initial stages of machine perfusion or perhaps the half-life is only extended in the context of a high NRF2 environment. Ultimately, the detailed mechanism of NRF2 regulation in both groups of livers and specifically why the high NRF2 group was able to further increase NRF2 levels while the low NRF2 group experience further protein loss remains to be explained. This could be related to the quality of the allografts although basic donor demographics were similar in both groups. An intriguing question is whether NRF2 needs to reach a certain threshold or cut off to benefit from further stimulation and larger samples would be needed to address this.

Owing to the fact that reperfusion is the main cause of injury in marginal livers, the current set of experiments also determines that livers with high NRF2 expression had substantially lower degrees of periportal inflammation. Specifically, livers with a high expression of NRF2 were able to maintain significantly lower inflammatory scores around the hepatic artery while there was greater inflammatory infiltration in low NRF2 livers. In a mouse model using static cold storage followed by liver transplantation, Ke et al found that Keap 1 ablation resulting in subsequent NRF2 increases reduced macrophage and neutrophil trafficking, pro-inflammatory cytokine release and hepatocellular death in livers during IRI [240]. Lower inflammatory cell infiltration could also be related to reduced chemotaxis in livers with high NRF2 expression levels. There is strong evidence in the literature linking high NRF2 levels to the release of pro-inflammatory cytokines, most commonly TNF- α [338-342]. TNF- α has significant chemotactic properties on NK-cell, neutrophils, monocytes and macrophages [343-345] and this could explain the increased inflammatory infiltration on both H & E and IF staining. In essence, livers with higher NRF2 appear to experience the benefits of NMP while livers with lower levels experience greater injury. This mechanism could be useful in determining which livers should undergo machine perfusion.

The ability of high NRF2 livers to clear lactate more efficiently than low NRF2 livers is an important clinical finding as these levels met viability criteria determined by the VITTAL trial [332]. Several reports have set transplant eligibility when a lactate threshold of ≤ 2.5 mmol is achieved, correlating with allograft viability and post-operative function [346-348]. The liver

plays an important role in lactate clearance and can remove 50% - 70% of circulating serum lactate [349, 350]. For these reasons lactate clearance can be viewed as a surrogate marker of hypoxic injury and hepatic function [351, 352]. There is logic to speculate that the role of NRF2 in antioxidant response and aerobic metabolism could influence blood lactate clearance; however, to date, no study has reported on this relationship directly which would be of immense value in the current liver machine preservation era [353-355].

Some notable molecular changes were also observed in our experiments in the context of NRF2. Our previous experiments showed significantly higher pAMPK in high NRF2 livers when compared to livers with lower NRF2 expression levels. Both high and low NRF2 groups decreased pAMPK levels following perfusion and this was most significant in the high NRF2 group at the 6 hour timepoint. The findings could be explained by the fact that tAMPK levels slightly increased between 0 and 6 hours in the high NRF2 group which could account for the significant decrease in pAMPK. A more substantial increase in tAMPK may have cancelled out this ratio and this appears to have occurred in the low NRF2 group. The influence of NMP itself likely plays a critical role in these molecular changes. Activation of the AMPK pathway during machine perfusion has been previously associated with better AST/ALT levels [313] and this could explain why high NRF2 livers experienced lower transaminase derangements and still maintained a slightly higher level of pAMPK at the end of NMP. This also supports the hypothesis that high NRF2 livers are more viable and are to maintain metabolic processes. The decreased levels, however, could be explained by the fact that like HO-1, AMPK phosphorylation relies on several other substrates for its activation and thus it is unlikely that NRF2 is the only critical factor [356-359]. Moreover, it could also be related to the metabolic potential and quality of the current sample of discarded livers that are likely still experiencing some level of injury during NMP.

In our experiments, NMP appeared to further drive NRF2 activation by decreasing Keap – 1 levels in the high NRF2 livers after 6 hours of machine perfusion. This supports the traditional mechanistic perspective that NRF2 is inhibited by Keap 1 and that when this is degraded, NRF2 levels are increased [360, 361]. The result also explains why we saw a higher nuclear intensity of NRF2 on IF staining in high NRF2 livers after machine perfusion. In livers with lower expression levels of NRF2, Keap-1 levels were grossly unchanged after NMP. The ability of some livers to respond positively to NMP could be explained by this

observation and the inability of a proportion of livers to degrade Keap 1 could mean that antioxidant mechanisms can't be activated in all samples [362].

Both groups of livers were able to increase pERK levels and this change was most significant in livers with low expression levels of NRF2. As mentioned in the previous chapter, ERK phosphorylation was used as a measure to determine liver degeneration and the current experiments appear to demonstrate the ability of NMP to do so. We previously demonstrated that livers with high NRF2 also demonstrated high expression of pERK and that this may support their ability to recover from oxidative stress and IRI. After NMP, livers with higher NRF2 expression maintained higher pERK expression than livers with lower NRF2, but this did not achieve statistical significance. Remarkably, livers with lower NRF2 expression increased pERK significantly between the 0 and 6 hour timepoints suggesting that pERK functions independently of NRF2 and demonstrating the ability of these livers to regenerate. However, this did not necessarily translate to better clinical parameters in these livers which still showed a greater degree of injury. This could also still relate to the bidirectionality of pERK and its ability to regulate cell proliferation implying that its activation may not always be viewed positively [316]. Encouragingly, CCP-3 which was used as a measure of cell death was decreased in both groups of livers. This finding may also be unrelated to NRF2 content and could be perceived more as a benefit of NMP and its ability to reduce cell death.

The novelty of the current experiments was that lean livers significantly increased NRF2 levels after 6 hours of NMP while steatotic livers significantly lost NRF2 levels. This aligns with previous research of the inverse relationship between NRF2 and steatosis, however, how this relates to NMP was not reported until now. These findings could add to the growing body of work attempting to explain why steatotic livers are more susceptible to IRI and why some may not experience the benefits of NRF2 [363, 364]. Interestingly, one of the livers in the high NRF2 group was declined for transplant because of steatosis. Combined with the current findings, this could suggest that steatosis itself does not denote marginality and that even some steatotic livers could be appropriate for transplantation, withstand IRI and benefit from NMP. Most importantly, the data could indicate that NRF2 activation may

be a novel pharmacologic target for marginal livers prior during NMP and ameliorate their susceptibility to injury.

4.6 Implications

What is already known:

The ongoing shortages of liver allografts have warranted the consideration of livers that would have been previously declined due to concerns about quality. Marginal livers are more susceptible to ischaemia reperfusion injuries during transplantation and for these reasons, many centers limit their use. The novelty of normothermic machine perfusion has made the utility of these allografts a reality and in some centers marginal livers have been “rescued” and reused for transplantation after a period of NMP. The advantage of NMP is in its ability to maintain cellular metabolic processes and therefore prevent the previous cold ischaemic injury of static cold storage, the traditional method of organ preservation.

What is new:

Livers with a high expression of NRF2 were able to benefit from NMP and demonstrated significantly less inflammatory injury after 6 hours of perfusion. High NRF2 livers also had significantly lower transaminase derangements and were able to clear lactate which is an important marker of viability currently used by NMP trials internationally. The ability of lean livers to increase NRF2 expression after NMP was also novel. The inability of steatotic livers to show a similar response after NMP may explain the susceptibility of some allografts to IRI and specifically the inability of steatotic livers to benefit from NMP.

Potential impact on future practice:

Some livers may be resistant to NMP and its protective influences making them unsuitable for transplantation. The ability of some livers to react positively by increasing NRF2 after NMP could help determine which livers are likely to benefit from the machine and ultimately reduce unnecessary liver discard. The data also suggests that not all steatotic livers are incapable of generating an NRF2 response and that these livers can also experience less injury during NMP making them suitable for transplantation. Reducing organ discard from both angles could expand the donor pool and potentially reduce wait list mortality.

Chapter 5

Strategies used to target NRF2 in liver surgery

5.1. Introduction

Tissue ischaemia is a major contributor of pathological disease states. The apparent paradox in ischaemia reperfusion injuries (IRI) is that severe molecular dysfunction and eventual cell death worsens following the restoration of blood flow to a previously ischaemic section of tissue [365]. In this scenario, while revascularization appears paramount and is a reasonable therapeutic option in nearly all instances, reperfusion can elicit further pathogenetic processes worsening injury, threatening organ functionality and leading to extensive tissue trauma. One of the main driving factors in cellular damage is the generation of high levels of reactive oxygen species (ROS) which modify protein and lipid macromolecules facilitating initiation of apoptotic and necrotic cascades through subsequent DNA mutations [366]. This results in downstream activation of inflammatory processes with diverse repertoires of cytokines and chemokines causing consequential parenchymal and sinusoidal injury [367]. Excessive ROS production also triggers a protective oxidative stress response in an effort to restore cellular redox balance. There is convincing evidence linking IRI with the NRF2 axis [243], representing a natural cellular response to oxidative stress by upregulating endogenous anti-oxidant genes to combat the ischaemic insult. Data reported within this thesis has already demonstrated significant variation in hepatic NRF2 protein expression in multiple discarded liver allografts. A proportion of liver discards had higher NRF2 protein expression than others and similar variability in the degree of inflammation was also demonstrated. This adds to current discussions on genetic variability in relevant transplant molecules and how these may impact patient outcomes. Additionally, identifying key immunoregulatory molecules can facilitate the development of logical individually tailored therapeutic targets.

Current concepts suggest that modulating the degree of oxidative stress may mitigate the inflammatory changes observed during IRI [368]. ROS are important mediators in cellular injury and contribute to the production of several proinflammatory cytokines including IL-1 β , IL-6, and TNF- α [369, 370]. Consequently, these cytokines facilitate the activation and recruitment of inflammatory cells contributing to the further generation of ROS (Figure 5.1). Amongst the mechanisms which may play a role regulating these pathways is NRF2 augmentation and this observation has since provoked the hypothesis that NRF2 targeting may have clinical benefits. Therapeutic NRF2 activation has been previously evaluated in

rodent models of retinal ischaemia by temporarily increasing intraocular pressures to mimic ischaemic states and subsequently normalize pressures to achieve vascular re-flow [265]. In these experiments, superoxide, proinflammatory mediators and leucocyte infiltration were greatly accentuated in NRF2 knockout mice when compared to a wild type control suggesting that pharmacologic induction of the axis could be a new therapeutic strategy in retinal diseases. The latter was also achieved in a rodent model of macular degeneration which was pretreated with CDDO-trifluoroethylamide [-TFEA] and resulted in retinal cytoprotection through NRF2 induction [371]. Using a similar concept, low concentrations of carbon monoxide were found to mediate NRF2 activation and offered an encouraging neuroprotective agent in the treatment of ischaemic stroke [264]. Moreover, the axis has also emerged as a promising pharmacologic target when combatting cardiac ischaemia emphasizing its potential role, not only as an effective biomarker, but a molecular target [267].

Although several studies support therapeutic NRF2 in ischaemic and IRI settings, the therapeutic impact of NRF2 modulation has not been fully translated clinically in liver surgery and specifically liver transplantation. Several experimental animal studies have highlighted some promising results in terms of IRI attenuation during liver transplantation by comparing wild type and knock out rodent models. The primary focus of the current chapter is to summarize the feasibility and utility of therapeutic NRF2 amplification in the management of IRI during liver transplantation surgery.

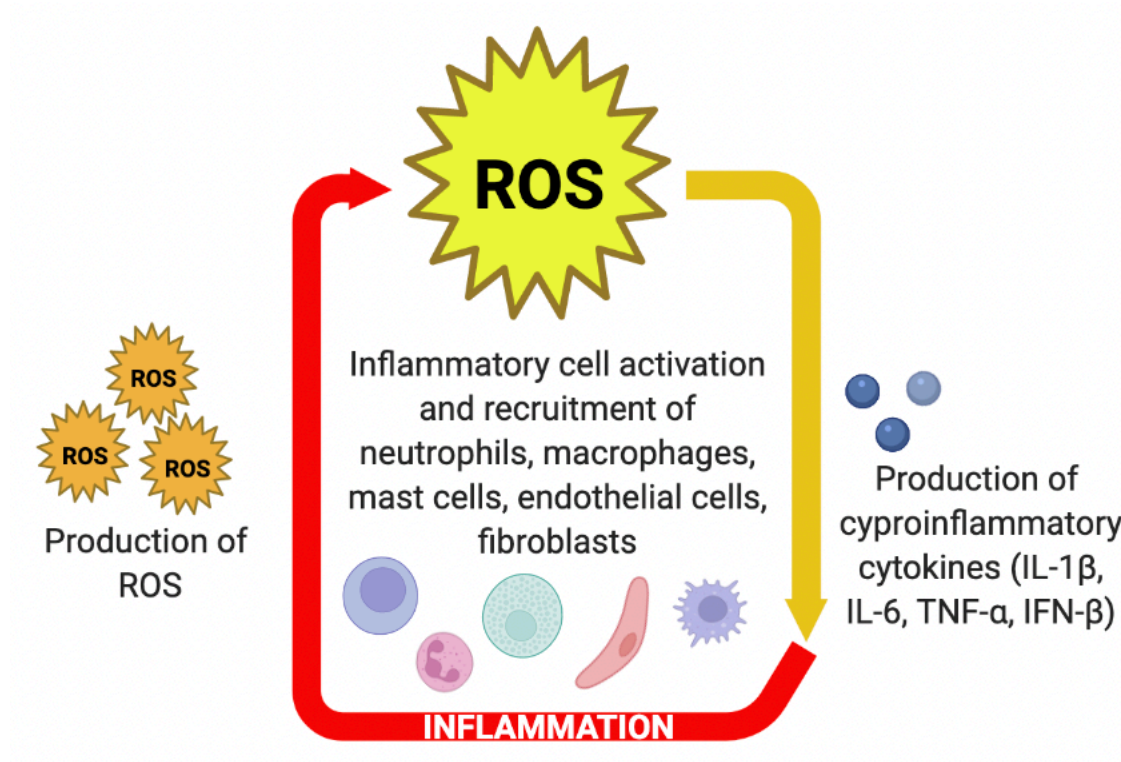


Figure 5. 1 *Reactive oxygen species (ROS) and inflammation*. ROS initiate inflammation through the production of pro-inflammatory cytokines as part of the immune process. This recruits immune cells to the site of injury which further produce high ROS levels leading to a cycle of continued inflammation if left unchecked in the context of oxidative stress. Both processes continue to aggravate the other leading to cell injury and tissue damage.

Hypothesis:

The NRF2 axis influences discarded allograft function and can be considered a biomarker for marginal liver selection.

Overall aim:

To study the role of hepatic NRF2 in allograft function, injury and repair systems.

5.2 Specific aims

The aims of the present chapter are to:

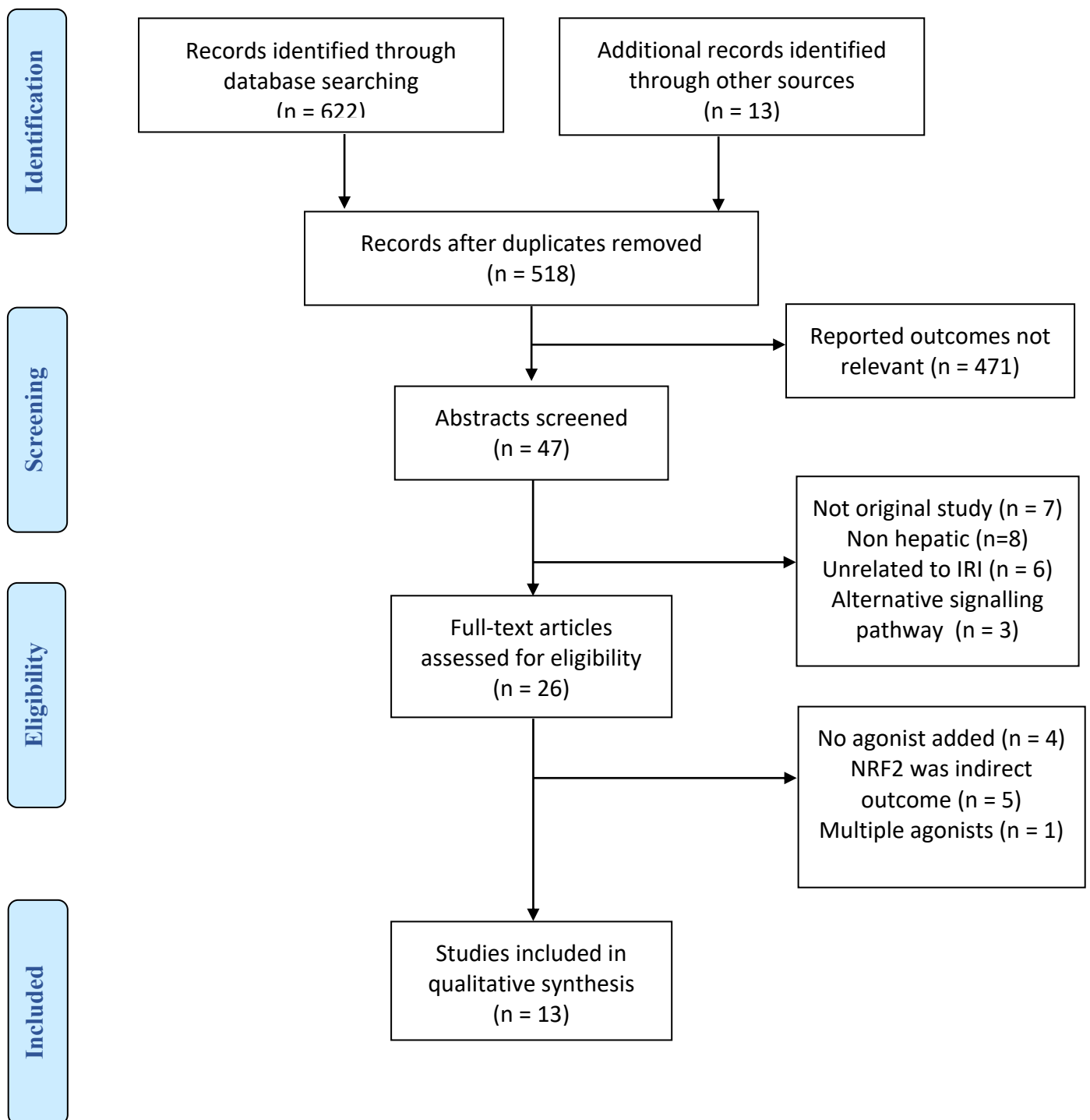
- Review the literature for studies in experimental hepatic IRI models with a special focus on NRF2 amplification.
- Summarize the impact of NRF2 activation on hepatic injury and function
- Determine where the weight of evidence lies with regard to the cytoprotective elements of exogenous NRF2 amplification and critically examine how this may relate to recent developments in the literature for hepatic tissue injury.

5.3 Results

5.3.1 Literature Search Results

In addition to the primary electronic search (as discussed in the methods chapter in section 2.2), the bibliographies of selected articles were manually reviewed to identify other studies for inclusion and an additional review of the “related citations” in PubMed was also performed. The literature search revealed 622 publications with a further 13 added from analyzed bibliographies. Following the exclusion of duplicate articles, 518 articles were screened and, ultimately, a total of thirteen studies met the criteria for inclusion in which the influence of synthetic and/or non-synthetic NRF2 amplification drugs were evaluated in hepatic IRI. The study selection process is illustrated in Figure 5.2.

Figure 5. 2 PRISMA flow Process for study selection



5.3.1 Study characteristics

All study designs included at least two groups, of which one received an NRF2 agonist and the other group was administered a vehicle control. Study characteristics are detailed in Table 5.1. There was relative heterogeneity in experimental design and few studies effectively removed their risk of bias based on assessment of the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) tool (Figure 5.3). Eight studies utilized a mouse model [339-342, 372-375] while the remaining five were rat models [338, 376-379]. Murine model ages ranged from 5 to 12 weeks [339, 340, 342, 372, 374, 376, 379]. An additional human cohort was included in one study in which the authors employ propofol post-conditioning in an effort to augment the NRF2 pathway [372]. Human participants were randomized into a treated group (n= 16) and a control group (n= 21) with no differences in patient demographics or diagnoses. Median ischaemia time was 60 minutes (range 30 – 90 minutes) at a median of 70% arterial/venous occlusion followed by reperfusion. Oxidative stress secondary to IRI resulted in endogenous NRF2 upregulation in control groups which were not treated with an agonist [339, 340, 372, 374-379]. Twelve study models induced NRF2 amplification prior to IRI at varied time points, except for one group in whom ischaemic conditioning was initiated 10 minutes post reperfusion [372]. Drug administration time points are presented in Table 5.2.

Table 5. 1 Studies using NRF2 upregulation during hepatic ischaemia reperfusion (n = 13).

Year	Author	Institution	Country	Animal model	Nrf2 agonist	Mechanism of action
2019	Rabie[338]	Cairo University	Egypt	rat	Telluric acid	ROS scavenger, anti-inflammatory action, NRF2 activation
2017	Zhou[375]	Guilin Medical University	China	mice	DADLE	Promotes NRF2 nuclear translocation
2017	Zeng[379]	Transplant Center of Wuhan University	China	rat	tBHQ	Induction of NRF2 pathway
2017	Xu[339]	Shanghai Jiao Tong University	China	mice	CDDO-Im	Activation of the NRF2/HO-1
2017	Chen[340]	Tongji University School of Medicine	China	mice	15d-PGJ2	NRF2 activation
2017	Ge[372]	Sun Yat-sen University	China	mice	Propofol	NRF2/HO-1 transcription
2015	Chi[376]	Sun Yat- sen University	China	rat	Sulfurane	NRF2 nuclear localization and ARE transactivation
2015	Joe[376]	University of Ulsan,	Korea	mice	Cilostazol	Upregulates HO-1 through NRF2 activation
2014	Shimada[376]	Hokkaido	Japan	mice	Sodium hydrogen sulfide	Phosphorylation of Akt and nuclear translocation of NRF2
2014	Kudoh[342]	Akita University Graduate School of Medicine	Japan	mice	15d-PGJ2	NRF2 activation
2014	Masuda[378]	Shinshu University	Japan	rat	Dh404	NRF2 nuclear translocation
2013	Park[373]	Sungkyunkwan University	Korea	mice	Nicotine	Nuclear translocation of NRF2
2012	Kang[377]	Sungkyunkwan University	Korea	rats	Melatonin	NRF2 nuclear translocation

DADLE: D-Ala2, D-Leu5-Enkephalin; DOR: δ -opioid receptor; tBHQ: Tert-butylhydroquinone; 15d-PGJ2: 5-deoxy- Δ 12, 14- prostaglandin J2; Dh404: CDDO-9,11-dihydro-trifluoroethyl amide; CDDO-Im: CDDO Imidazole

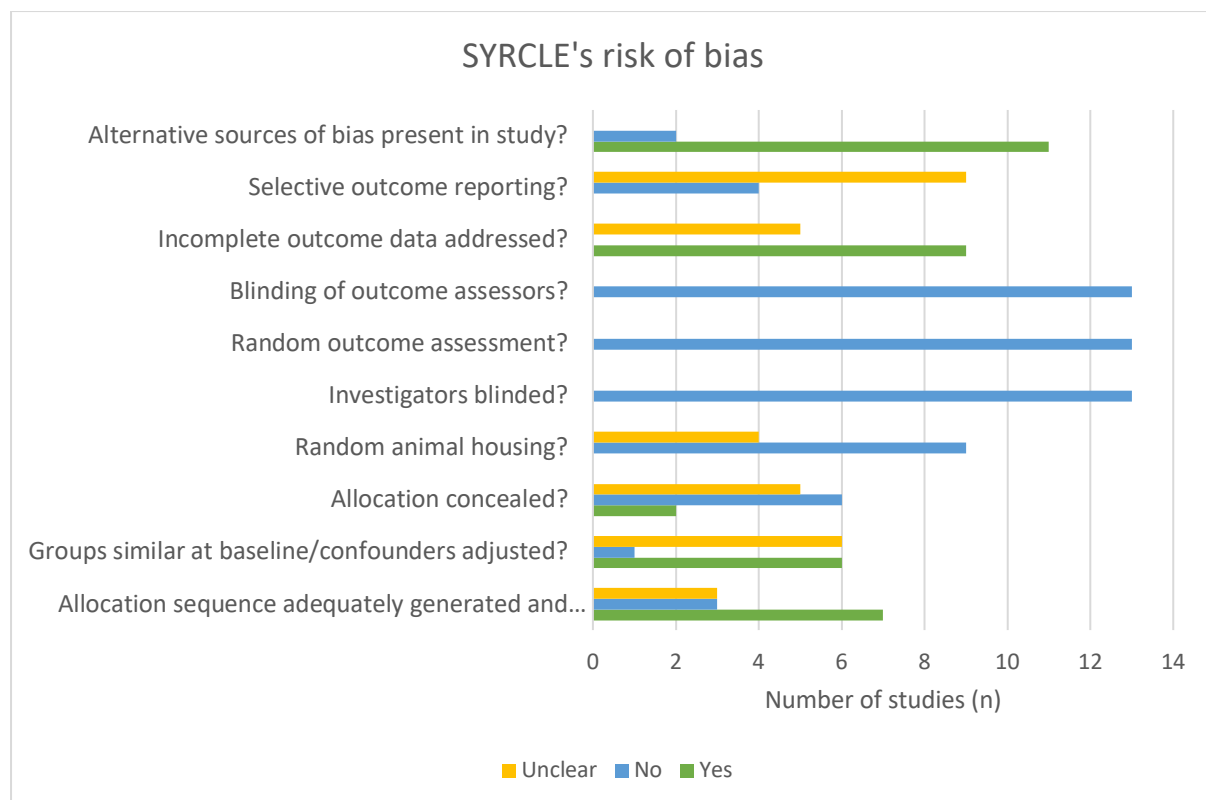


Figure 5. 3 SYRCLE's risk of bias tool for animal studies. This is a widely used, validated risk of bias used in the analysis of animal intervention studies [380]. The tool consists of 10 entries which consider 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and other biases. Most of the current studies experienced bias due to lack of blinding and inadequate randomization of animals. Additionally, several studies reported their data in an incomplete manner despite outlining their design in the methodology.

5.3.2 Liver function

Serum enzyme assays were evaluated in all studies following periods of hepatic IRI and resulted in markedly reduced enzyme levels in treated groups. Nine studies included assessments of both ALT and AST levels [338-340, 342, 372, 374-376, 379], with only four studies reporting ALT assays [341, 373, 377, 378]. Serum markers were measured at individual time points of (median) 6 hours (range 2 – 24 hours) [338, 339, 341, 342, 373-379] post reperfusion in all studies and at multiple time points in two further studies (Chen et al at 6, 12, 24 hours [340]; Ge et al at 6 hours, 24 hours, 2, 3, 5, 7 and 14 days [372]). In two studies, positive dose dependent responses were observed with lower levels of serum enzyme activity at higher doses of medication. Serum ALT was markedly reduced to 1514 ± 159 U/l and 1334 ± 180 U/l following 0.3 and 1mg/kg of nicotine [373]. Similarly, varying degrees of protective effects were observed with 2.5 μ g, 7.5 μ g and 15 μ g dosages of 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), with the highest dose attenuating injury and maintaining the protective impact at 6, 12 and 24 hours ($P < 0.05$) [340]. Serum ALT levels were explicitly outlined in four manuscripts, improving from a mean of 2704.1 ± 403.1 to 1132.5 ± 228.7 post perfusion when the medication was administered [373, 377-379]. Similar improvements were demonstrated in a human group receiving propofol post conditioning [372]. The treated cohort demonstrated a faster return to AST levels within 7 days ($p = 0.012$) and ALT levels within 30 days (0.019). The same group also achieved a quicker resolution of prothrombin time within 3 days ($p = 0.006$) and hyperbilirubinemia within 14 days ($p=0.002$) in the post-operative period.

Table 5. 2 Timing of pharmacologic NRF2 augmentation in selected studies.

Year	Author	NRF2 agonist	Timing of administration
2019	Rabie[338]	Telluric acid	1 week of treatment before surgery
2017	Zhou[375]	DADLE	15 minutes before IRI
2017	Zeng[379]	Tert-butylhydroquinone (tBHQ)	12 hours before IRI
2017	Xu[339]	CDDO-imidazolidine	3 hours before IRI
2017	Chen[340]	15d-PGJ2	30 minutes before IRI
2017	Ge[372]	Propofol	Immediately following reperfusion
2015	Chi[376]	Sulforane	1 hour before IRI
2015	Joe[376]	Cilostazol	3 days of treatment before IRI
2014	Shimada[376]	Sodium hydrogen sulfide	10 minutes before IRI
2014	Kudoh[342]	15d-PGJ2	3 hours before IRI
2014	Masuda[378]	Dh404	5 hours before IRI
2013	Park[373]	Nicotine	20 minutes before IRI
2012	Kang[377]	Melatonin	15 minutes before IRI

DADLE: D-Ala², D-Leu⁵-Enkephalin; DOR: δ -opioid receptor; tBHQ: Tert-butylhydroquinone; 15d-PGJ2: 5-deoxy- Δ 12, 14- prostaglandin J2; Dh404: CDDO-9,11-dihydro-trifluoroethyl amide; CDDO-Im: CDDO Imidazole; IRI : ischaemia reperfusion injury

5.3.3 Anti-oxidant expression

Although NRF2 expression was successfully induced in 13 studies using exogenous drug administration, only 11 studies directly measure NRF2 expression [338-341, 372-377, 379]. Seven studies specifically measured and reported an increase in nuclear NRF2 expression that was captured on western blotting analysis in all and in both western blotting and immunofluorescent staining in one study [372]. One paper did not account for direct NRF2 expression when using an agonist and only measured downstream representative target genes of NRF2 as an indication of its activation post drug administration [342]. One additional paper failed to see a significant increase in NRF2 expression on both nuclear and cytosolic contents using western blotting [378]. Table 3.3 details the changes to NRF2 for each of the selected papers.

Surrogate markers of NRF2 activity were recorded in all studies, most commonly HO-1 which was upregulated in treatment groups prior to [339, 341, 373-377, 379] and following ischaemia reperfusion injury [372]. Increased HO-1 activity was expressed on western blotting of treated and untreated hepatic tissue. Numerical data was explicitly defined in two manuscripts in which HO-1 activity increased from 51.9 ± 5.55 to 79.2 ± 6.05 pmol bilirubin/min/mg ($p < 0.01$) within pretreated groups following exposure to ischaemia and reperfusion [373, 377]. Participants subjected to hepatic ischaemia also demonstrated marked elevations in antioxidant activity in terms of GSH levels [338, 373, 375], NQO1 [339, 342, 372, 376, 379], Gclc [342, 378, 379], GSTm1 [339, 342], CXCL 10 [339, 377] and TRX-1 [374].

Table 5. 3 Measure and expression of NRF2 in selected manuscripts

Author	Changes to NRF2	Method used
Chen	Increased nuclear expression	Western blot
Chi	Increased expression	Western blot
Ge	Increased nuclear expression	Western blot and immunofluorescent staining
Joe	Increased nuclear expression	Western blot
Kang	Increased nuclear expression	Western blot
Kudoh	Not directly measured	-
Masuda	No difference in nuclear or cytoplasmic expression	Western blot
Park	Increased nuclear expression	Western blot
Rabie	Increased expression	Western blot
Shimada	Increase nuclear expression	Western blot
Xu	Increased nuclear expression	Western blot
Zeng	Increased expression	PCR
Zhou	Increased nuclear expression	Western blot

5.3.4 Anti – inflammatory activity

Seven studies included data on pro and anti-inflammatory cytokine activity [338-342, 373, 374, 377]. Ischaemia reperfusion injuries were largely associated with higher serum levels of inflammatory activity which improved significantly when subjects were pre-treated. Serum TNF- α levels were markedly reduced in seven studies [338-342, 374, 377]. Therapeutic NRF2 amplification was also inversely correlated with reduced interleukin – 1 beta [339, 340, 374], interleukin - 6 [339, 374], interleukin - 23 [374] and interferon-gamma [374] and beta [377]. Inflammatory alterations are summarized in Table 3.4

Table 5. 4 Inflammatory mediator responses following NRF2 augmentation during IRI.

Year	Author	Nrf2 agonist	Inflammatory activity
2019	Rabie[338]	Telluric acid	↓ TNF- α
2017	Xu[339]	CDDO-imidazolide	↓ TNF- α , ↓ II – 1 β , ↓ II -6
2017	Chen[340]	15d-PGJ2	↓ TNF- α , ↓ II – 1 β
2015	Joe[376]	Cilostazol	↓ TNF- α
2014	Shimada[376]	Sodium hydrogen sulfide	↓ II – 6, II – 1 β , ↓ IFN β , ↓ II-23
2014	Kudoh[342]	15d-PGJ2	↓ TNF- α
2013	Park[373]	Nicotine	↓ TNF- α
2012	Kang[377]	Melatonin	↓ IFN β

5.3.5 Histology

Histopathological grading of treated and control liver sections was performed as described by Suzuki et al [381] in twelve studies [338-340, 342, 372-379]. Groups treated with NRF2 amplifying medication largely demonstrated lower Suzuki scores, reduced areas of cellular infiltration and histologic features of ischaemic damage (Table 5.5).

Table 5. 5 Histological changes during NRF2 augmentation and hepatic IRI

Author	Treatment	Ischaemic time (minutes)	Histology
Rabie[338]	Telluric acid	30	Maintained histological structure of central vein and surrounding hepatocytes.
Zhou[375]	DADLE	45	Reduced hepatocellular swelling and inflammatory infiltration
Zeng[379]	tBHQ	60	Less oedema, inflammatory cell infiltrate, apoptosis, and necrosis
Xu[339]	CDDO-imidazolid	90	Decreased cellular infiltration
Chen[340]	15d-PGJ2	60	Reduced hepatocyte necrosis and organelle swelling
Ge[372]	Propofol	60	Less hepatocyte swelling, vacuolar degeneration, necrosis, sinusoidal congestion, and inflammatory cell infiltration
Chi[376]	Sulfurane	60	Less vacuolar degeneration, neutrophil infiltration, patchy necrosis, sinusoidal narrowing, swelling

Shimada[376]	Sodium hydrogen sulfide	75	Less inflammatory infiltration, congestion, vacuolization
Kudoh[342]	15d-PGJ2	60	Less cell swelling, karyolysis, loss of basophilic staining, and vacuolization
Masuda[378]	Dh404	60	Less sinusoidal congestion, vacuolization, and focal necrosis
Park[373]	Nicotine	60	Less inflammatory cell infiltrate and less cellular congestion
Kang[377]	Melatonin	60	Less portal inflammation, hepatocellular necrotic lesions, inflammatory cell infiltration and congestion

DADLE: D-Ala2, D-Leu5-Enkephalin; DOR: δ -opioid receptor; tBHQ: Tert-butylhydroquinone; 15d-PGJ2: 5-deoxy- Δ 12, 14- prostaglandin J2; Dh404: CDDO-9,11-dihydro-trifluoroethyl amide; CDDO-Im: CDDO Imidazole.

5.4 Discussion

The aim of this bibliographic review was to critically evaluate the evidence surrounding exogenous and therapeutic NRF2 intervention strategies in hepatic ischaemia reperfusion injury in the context of liver transplantation and allograft priming. The results herein summarize the biochemical, inflammatory and histological outcomes revealing some benefits in therapeutic anti-oxidant pathway amplification either prior to or immediately following ischaemic injury. Despite the broad differences in study design, IRI models and treatment protocols, the results could indicate a unique opportunity in the perioperative period to mitigate the physiological stress incurred by ischaemia reperfusion injuries during liver transplantation.

One of the many challenges of initiating effective treatment approaches is the complicated interplay of several mechanisms in the IRI process and a consequential cascade having the ability to impair multiple organs, not just limited to liver injury [382]. Several important pathways are implicated including anaerobic metabolism, Kupffer cell activity and oxidative stress processes [383]. It is widely recognized that the oxidative stress incurred by IRI results in ROS generation, the subsequent nuclear translocation of NRF2 and a powerful anti-oxidant response through this transcription factor [384]. One important consequence of NRF2 release is the upregulation of HO-1. There is strong evidence to support the clinical relevance of HO-1 cytoprotection and high hepatic expression post-transplant seems to correlate with better hepatocellular function and less cellular damage [385]. The current study similarly demonstrates the integral role of HO-1 in liver injury with several agents indirectly promoting its release in cellular damage. It may be a plausible thesis to further amplify this process in a clinical setting through endogenous or exogenous means to dampen intra- and post-operative oxidative stress and inflammation. Encouragingly, NRF2 amplification resulted in downstream anti-inflammatory responses during the IRI cycle. All studies that measured inflammatory cytokine activity post NRF2 treatment reported significant reductions in pro-inflammatory cytokine activity such as TNF- α , IL – 1 β , IL -6 and IFN- β , supporting the role of NRF2 not only in antioxidant gene expression but also in anti inflammatory processes.

Liver transplantation is an exemplar model of complex major surgery and, despite major advances in operative technique and graft survival, the risk of ischaemia reperfusion injury still remains a major concern [214, 386]. Effective strategies preventing IRI are still lacking and this could be in part due to the complex repertoire of inflammatory and metabolic mechanisms implicated in the process [197, 387]. Many interventions have been proposed in an effort to decrease IRI, but few have been successfully translated into modern clinical practice [388-390]. Simillis et al attempted to consolidate evidence from several studies and trials incorporating several strategies aimed at decreasing IRI during elective hepatectomy [391]. Interventions included hypothermia, ischaemic pre-conditioning, antioxidants, immunomodulators, cardiovascular modulators, steroids, methods to increase hepatic glycogen and other miscellaneous therapies. In comparing 8 published measures from 44 trials used in 2,457 patients, all were similar with respect to mortality, blood transfusion and length of ITU stay, but no comparison of parenchymal injury in terms of serum or histological analysis was interpreted. The authors concluded that ischaemic pre-conditioning may be beneficial in this setting, offering the most clinical benefits in terms of less blood loss and length of stay when compared to other interventions or no intervention at all.

There was some heterogeneity in ischaemia reperfusion models among the included studies, with some using 30 minutes of ischaemic time and others reporting up to 90 minutes prior to reperfusion. Such discrepancies may exert different degrees of hypoxic damage, resulting in various levels of reperfusion injury. On that account, in order for reperfusion injury to occur, tissue must be ischaemic and not only hypoxic avoiding a common misnomer of IRI. It is unclear in several studies in the literature whether ischaemic injury has occurred and more practical parameters clinically characterizing degrees of ischaemia in a surgical setting are warranted. The need for accurate reporting of previously validated and standardized nomenclature is evidently needed to avoid the misjudgment of ischaemic injury and to help propose realistic therapeutic strategies in liver surgery. Such events are even more critical in fatty or marginal livers which are likely more vulnerable to ischaemia reperfusion injuries and therefore rely on accurate measures of ischaemic damage. Additionally, no study reports on prior cold ischaemia time. There is strong evidence in the transplant literature regarding the implications of prolonged cold ischaemia times (CIT) leading to early allograft dysfunction [392, 393]. This is even more consequential

when marginal livers or DCD donors are considered and the general guidance is that these livers should be transplanted within 8 - 12 hours of CIT to minimize allograft dysfunction[393-395].

Considering the evidence to date, NRF2 amplification appears to be positively associated with less hepatocellular injury when initiated either before or immediately after IRI. That said, whether the association is cause or effect, it seems counterintuitive not to consider these treatment modalities and fully explore these options prior to their application in real-life clinical situations. One potential bias in this review is the utility of normal livers in the murine models. The increased acceptance and transplantation of marginal models makes this a glaring limitation and warrants further exploration in this context. The livers also were not transplanted and thus the true extent of the in vivo effects of NRF2 amplification cannot be concluded. Additionally, we cannot conclude that manipulation of the NRF2 pathway may not incur long-term effects particularly in a vulnerable, immunosuppressed recipient population. The studies also included animals from various age groups which may alter outcomes as increased age is a known risk factor for low NRF2 expression [247]. The data included is interpreted from small studies using a variety of enhancers. Some of the medications used have clear downsides, such as nicotine, with little to no potential for clinical crossover. Moreover, the review is also limited by the quality of the included studies. Although all the studies show significant improvement in physiological and pathological outcomes, post-operative and long-term outcomes are unclear and should be examined in well-designed prospective trials. Encouragingly the data presented could serve as a springboard to further investigate the therapeutic opportunity of NRF2 enhancement in mitigating IRI and were used to guide the final chapter of the current thesis.

This analysis suggests that several therapeutic agents can upregulate NRF2 in hepatic tissue, supporting concurrent evidence that the axis is a master regulator of various cellular processes. This, however, is not without consequence. While decades of research have confirmed that increased NRF2 activity is beneficial, there is increasing evidence correlating hyperactivity with tumor growth and dissemination in some organs [396]. It is now increasingly clear that several organ systems have catalogued NRF2 alterations in their cancer sequencing [397, 398]. An interplay between NRF2 and hypoxia inducible factor-1

(HIF-1) has been previously recognized in the context of the latter's role in tumor angiogenesis [399]. At a hepatocellular level, HIF-1 α attenuates hepatic inflammation following IRI exposure as a consequence of the upstream activation of NRF2 [384]. Several of the included studies utilized NRF2 amplification methods during the immediate perioperative period, except one in which the medication was administered for one week [338]. In this context, malignant implications may not play a role especially if subjects are treated in a "one-off" session, however, patients need close observation. Research in liver transplant surgery, oxidative stress, cancer development and possible common links should be explored using long-term analyses to consider the potential for malignant transformation.

The NRF2 antioxidant pathway is undoubtedly important in IRI attenuation, the exact therapeutic potential of this at a molecular level within liver surgery needs to be described and warrants further clinical attention. It may, however, remain difficult to truly establish whether NRF2 activation itself is an independent player without applying direct enhancers. How this is done is debatable. Novel therapies such as oltipraz and bardoxolone could be promising and subsequent analyses could compare various agents in comparative groups. As stated previously, IRI is a complex pathophysiological event involving the interplay of several inflammatory, antioxidant and metabolic factors. The ability of NRF2 to stimulate an improved physiological response makes it a potential and interesting target in the treatment of IRI.

5.5 Implications

What is already known:

Ischaemia reperfusion injuries (IRI) are caused by multifactorial processes which can lead to profound cellular damage and ultimately affect liver allograft function. The accruing evidence indicates that this can affect transplantation outcomes, predisposing allografts to acute/chronic rejection, organ dysfunction and primary non function. Identifying specific molecular mechanisms and pathways during IRI are therefore essential to mitigate the degree of injury and improve transplantation outcomes. The generation of reactive oxygen species triggers a delicate inflammatory cascade during which the NRF2 axis attempts to maintain redox balance and prevent cell death.

What is new:

The NRF2 pathway can be upregulated using a number of pharmacologic agents that modulate different phases of the axis. These can be administered immediately before or after the IRI occurs, and in some instances, several days before surgery. Augmentation in the liver prevents injury which has several functional effects including lower transaminases and pro-inflammatory cytokines as well as less inflammatory cell infiltration into the allograft. There was also an increase in downstream anti-oxidant genes such as HO-1, Gclc, and NQO1.

Potential impact on future practice:

Data from these studies suggests that NRF2 might be a good marker for hepatic function and resistance to IRI. Moreover, drugs that modulate NRF2 activity could be of value in pre-treated liver allografts by ameliorating the effects of IRI. Different phases of the axis could be targeted resulting in a reduced inflammatory response within the allograft and preserve its function. Organ shortages have prompted an interest in using extended criteria or marginal livers allografts to expand the donor pool. Such organs are more susceptible to IRIs and are used with caution. Efforts to optimize allograft function and prevent IRIs during the procurement and implantation process has the potential to increase the availability of allografts for transplant. These findings helped inform the final part of this thesis. They have strengthened the rationale for the hypothesis that NRF2 levels might be of prognostic use and might also provide a therapeutic target.

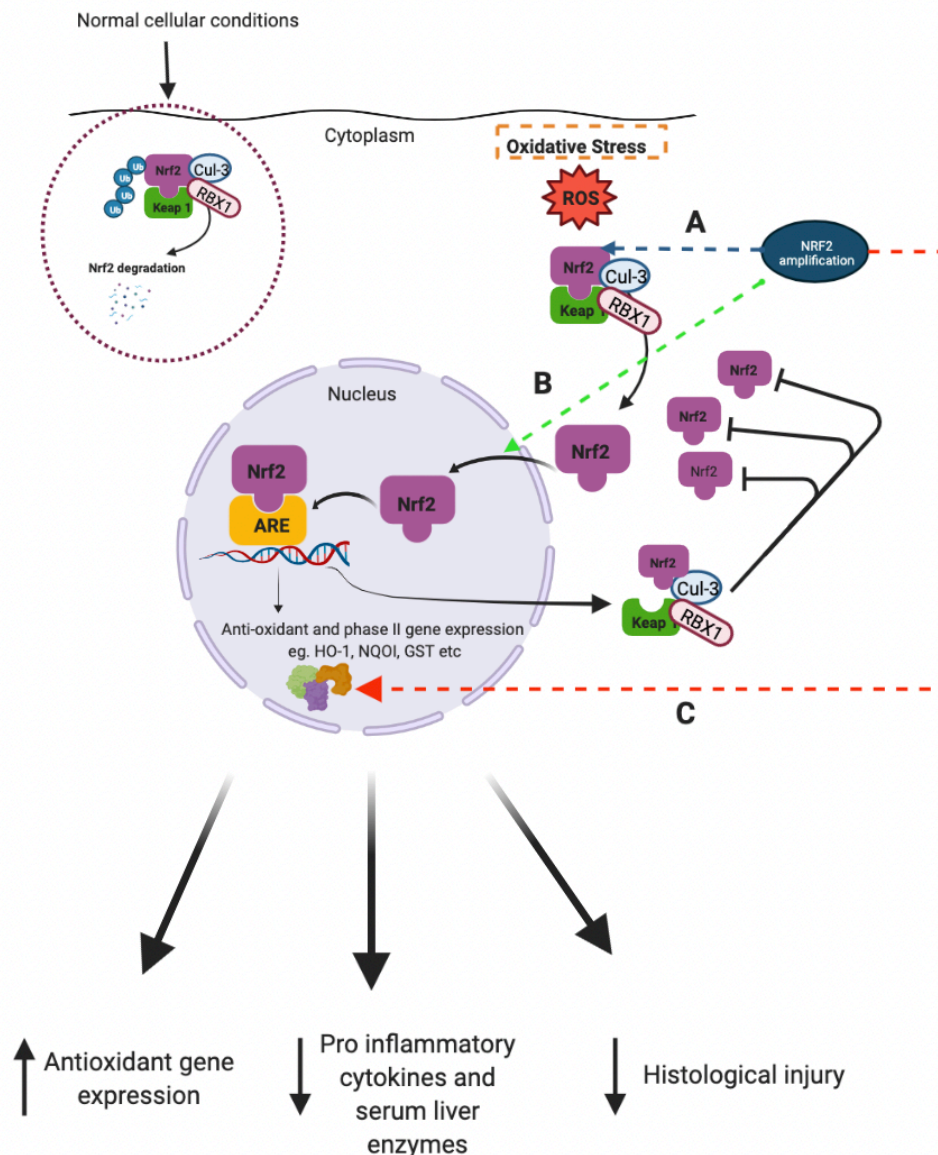


Figure 5. 4 *Graphical summary of NRF2 augmentation in liver allografts and therapeutic drug targets.* Therapeutic targeting of NRF2 appears to occur at three levels based on the findings of the literature review. **A)** NRF2 can be activated directly to amplify its antioxidant and anti-inflammatory effects, **B)** agonists can facilitate and increase the nuclear translocation of NRF2 and thus enable its binding with the antioxidant response element (ARE) and **C)** the downstream genes of NRF2 can be directly targeted to exert their anti-oxidant and anti-inflammatory effects. Ultimately, NRF2 amplification can increase antioxidant gene expression, decrease the expression of pro-inflammatory cytokines and subsequently decrease histological injury of the liver parenchyma during oxidative stress.

Chapter 6

NRF2 modulation in fatty livers during normothermic machine perfusion

6.1 Introduction

In the UK, previously declined liver graft offers contribute to almost a quarter of annual transplant activity [400] further driving the hypothesis that marginal livers and discarded livers can play an important role in expanding the donor pool. The quality of allografts in modern times comes into question and rising obesity rates have brought marginal liver allografts to the forefront. Orman and colleagues predicted that by the year 2030, the proportion of donors ≥ 50 years old will increase from 39.2% to 43.7%, the donor diabetic rate will increase from 13.8% to 45.7% and the prevalence of obesity among the donor pool will increase from 31.2% to 58.2% [37]. All of these factors would naturally contribute to the rising rate of NAFLD globally. A study by Eurotransplant aimed to assess changes to the donor pool in recent years and found that over 70% of liver allografts are transplanted after going through a secondary allocation and 26.6% are discarded [401]. The data also reported that nearly half of discarded livers were steatotic despite an increase prevalence of steatosis in livers accepted for transplant which rose from 22.3% to 51.5% in the 6-year study period. Miervini and colleagues studied 284 potential liver donors with an average age of 36.8 years and a BMI of 26.6 Kg/m² and found that only 42% had normal liver biopsies. Over 50% showed features of steatosis or steatohepatitis which led to the automatic disqualification of 3 – 21% of potential donors [402]. As such, the relatively indolent nature of NAFLD and its considerable ubiquity means that salvaging steatotic livers could contribute significantly to the current donor pool and address organ shortages.

As reiterated repeatedly in the preceding chapters, steatosis increases the susceptibility of livers to IRI due to lower ATP stores making them intolerant to ischaemia and the “reflow paradox” of an intricate inflammatory cascade involving antioxidant defences, pro inflammatory cytokines and chemokines [193, 329]. An additional unique concern in the use of steatotic livers is the impaired hepatic arterial flows and microcirculation ultimately affecting tissue oxygenation and exacerbating IRI [403]. Historical reports on experiences with steatotic livers for transplantation have consistently shown increased rates of early allograft dysfunction and primary non function [404, 405]. Additionally, steatosis is considered an independent risk factor for the development of biliary complications post-transplant [406]. This was in contrast to a recent study by Wong and colleagues in Hong Kong who successfully transplanted 19 severely steatotic liver allografts and reported a 1

and 3 year overall survival greater than 90% in their patient cohort [407]. While their routine use has yet to become mainstream, several strategies have been proposed to optimize outcomes especially during allograft preservation. Promising results by Zaouali et al showed that improving cold preservation storage for steatotic livers with the addition of melatonin and trimetazidine resulted in less ischaemic injury in steatotic allografts through AMPK phosphorylation and reducing ER stress [408]. The same group also showed that preserving livers at normothermic temperatures using insulin like growth factor-1 (IGF-1) enriched solutions improved steatotic liver preservation and inhibited the release of proinflammatory cytokines [409, 410]. More recently, Xu et al from Washington University School of Medicine in St Louis developed a novel multidrug defatting cocktail that was supplemented into NMP perfusate and treated livers experienced increased metabolism and less hepatocyte injury [320]. These results were similar to Boteon and colleagues who used a combination of drugs to enhance lipid metabolism in steatotic allografts during NMP to reduce reperfusion injury [411].

Given that a proportion of discarded livers remain functional, select allografts are able to withstand IRI by benefitting from NMP if the NRF2 axis remains intact. Chapter 4 specifically showed that some steatotic livers are able to maintain NRF2 expression levels and that this may reduce their susceptibility to injury. Encouragingly, Chapter 5 emphasized the potential for pharmacologic NRF2 targeting to reduce hepatic injury during IRI. A limitation of the included studies was their use of normal/lean liver models. The focus of this thesis thus far has been on marginal liver allografts and their responses during IRI. Considering that steatotic livers appear to have impaired and reduced NRF2 levels, modulating these levels may pre condition livers and reduce injury during transplantation. The literature was studied for an NRF2 agonist with a satisfactory therapeutic potential in marginal animal subjects. Oltipraz, [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione], is a synthetic dithiolthione initially developed for the treatment of schistosomiasis [412] and further pharmacokinetic studies demonstrated its ability to upregulate the NRF2 complex preventing the development of oxidative stress[413]. This molecule has been shown to have anticarcinogenic effects in experimental models in breast, bladder, liver, stomach, colon and lung cancers and is metabolized by the liver[414]. The advantage of utilising this medication in the current context was that several studies demonstrated both its safety and efficacy in minimising

oxidative injury in NAFLD, cirrhosis and diabetic subjects [412, 413, 415]. Additionally, our rodent NMP model is based on a 4-hour ex vivo perfusion strategy and thus requires a drug which is dissolvable in blood perfusate, metabolized by the liver and with an acceptable half-life and Vmax. Encouragingly, pharmacokinetic studies in rodent models showed that Oltipraz was rapidly absorbed and mean time to peak ranged from 100 to 140 minutes[416, 417]. This chemical profile made it feasible to use in the current model.

Hypothesis:

NRF2 amplification can offer another layer of protection for steatotic livers undergoing ex vivo machine preservation using normothermic techniques.

6.2 Specific aims

The aims of this chapter include:

- Inducing NRF2 activity in a murine fatty liver model using an NRF2 agonist
- Assessing the antioxidant outcomes of NRF2 augmentation during NMP
- Evaluate steatotic allograft function after NRF2 modulation.

6.3 Results

6.3.1 NRF2 augmentation in hepatic steatosis

Sprague Daley rats were fed a 60% high fat diet for 8 weeks to induce hepatic steatosis. Livers were procured and placed on an NMP model, similar to the human system. An NRF2 agonist was injected directly into the liver perfusate following the 1-hour mark to ensure allograft delivery and reduce systemic loss of drug (Figure 6.1). Animals were weighed weekly to ensure an increase in body weight and this was higher than the control group (Figure 6.2).

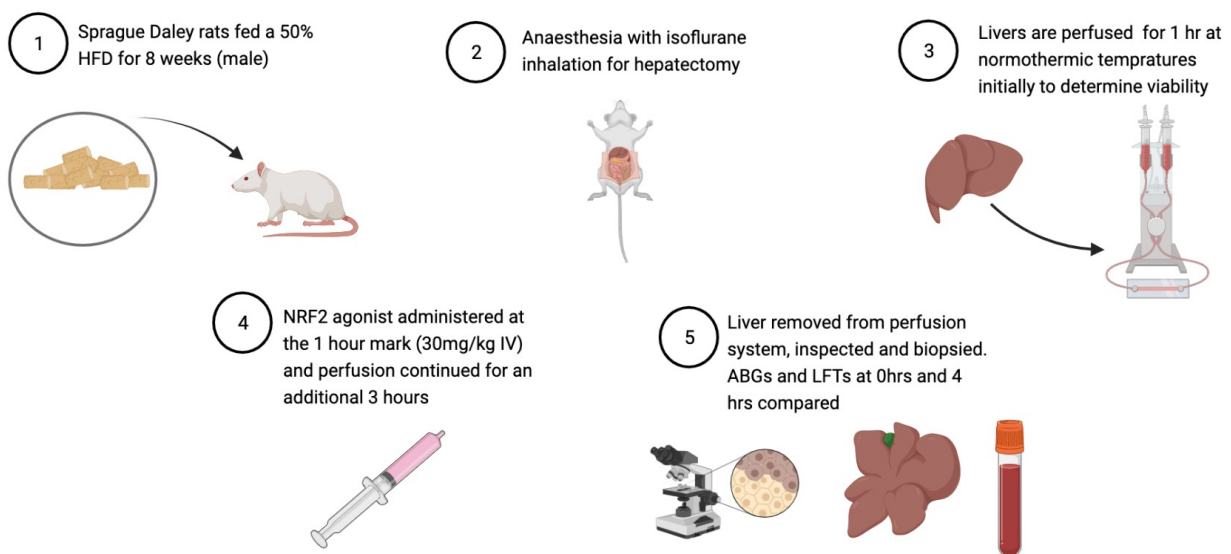


Figure 6. 5 Rat liver protocol for NRF2 amplification.

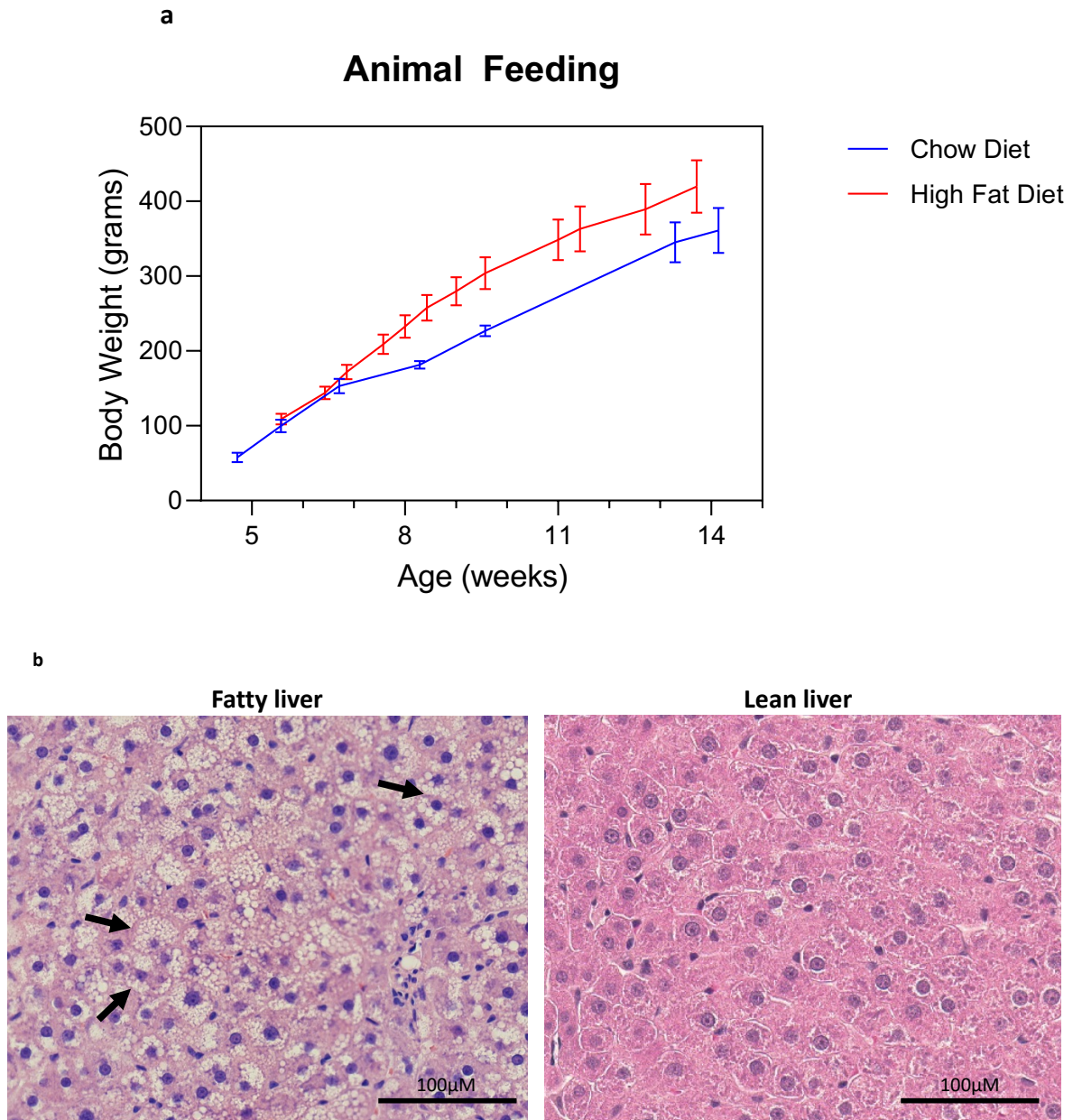


Figure 6. 6 *Rat liver steatosis after a high fat diet protocol.* **a)** Representative body weight curves in Sprague Daley rats after an 8-week high fat diet compared to a lean control group receiving a chow diet. **b)** Representative H&E staining of steatotic rat livers (arrows indicating areas of macrosteatosis) following an 8-week high fat diet and lean livers after a chow diet during a similar time frame.

6.3.2 Oltipraz treatment increased NRF2 translocation

The nuclear/cytoplasmic ratio was calculated in liver biopsies using IF at 0 hours and again after 4 hours of perfusion. Increased nuclear NRF2 intensity was observed in NRF2 treated rat livers when compared to the control group ($p = 0.043$) (Figure 6.3) and this was indicative of increased nuclear translocation.

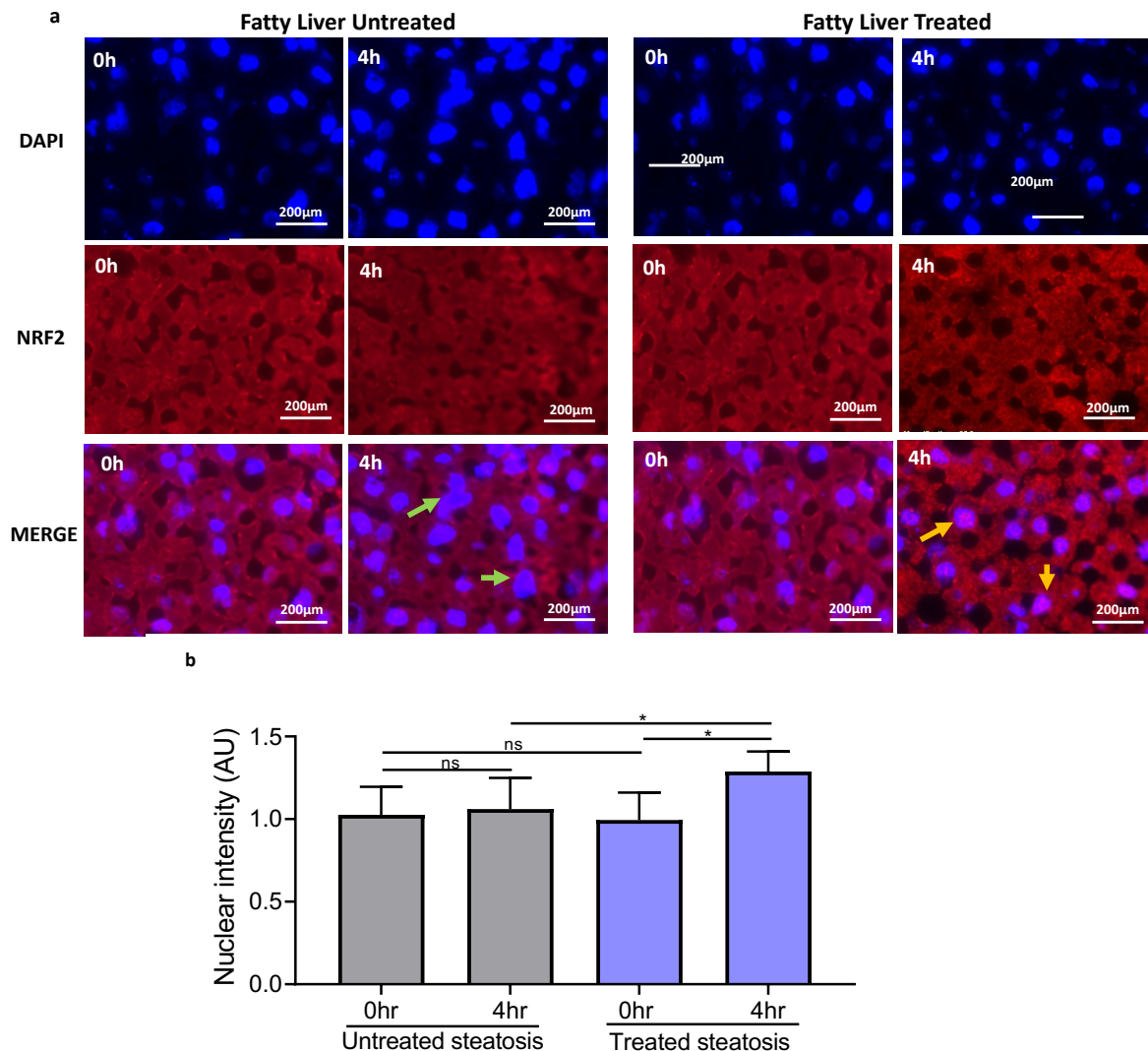


Figure 6. 7 *Immunofluorescent staining of steatotic rat livers before and after normothermic machine perfusion.* **a)** Increased nuclear intensity was observed in treated fatty livers (orange arrows) with few changes in untreated rat livers (green arrows) on quantitative analysis. **b)** Untreated steatotic livers had comparable nuclear levels of NRF2 at 0 and 4 hours of machine perfusion, while livers treated with NRF2 agonist had increased nuclear NRF2 expression after 4h NMP. AU = arbitrary unit. Data are represented as mean $\pm$ SD. *, $p < 0.05$; NS = not statistically significant.

6.3.3 Oltipraz treatment increased antioxidant gene expression

A significant decrease in NRF2 levels was observed in both treated ($p = 0.04$) and untreated steatotic ($p = 0.002$) rat livers after 4 hours of NMP despite both livers having equivalent NRF2 levels pre-NMP ($p = 0.114$). In untreated control livers, this was a -22% drop in NRF2 levels compared to on -5% loss in NRF2-agonist-treated steatotic livers and treated livers still maintained a significantly higher level of NRF2 after 4 hours of NMP ($p = 0.004$) (Figure 6.4a). Interestingly, at the 4 hour time-point, NRF2 levels in treated fatty livers were comparable to lean liver NRF2 levels. There was a significant parallel increase in HO-1 ($p = 0.016$) mRNA expression in treated livers following 4 hours of NMP when compared to untreated livers at the same time-point (Figure 6.4b). Additionally, while Gclc levels were observed to decrease in both groups of steatotic livers, the treated fatty liver group still maintained a significantly high level after 4 hours of machine perfusion Gclc ($p = 0.049$) (Figure 6.4c).

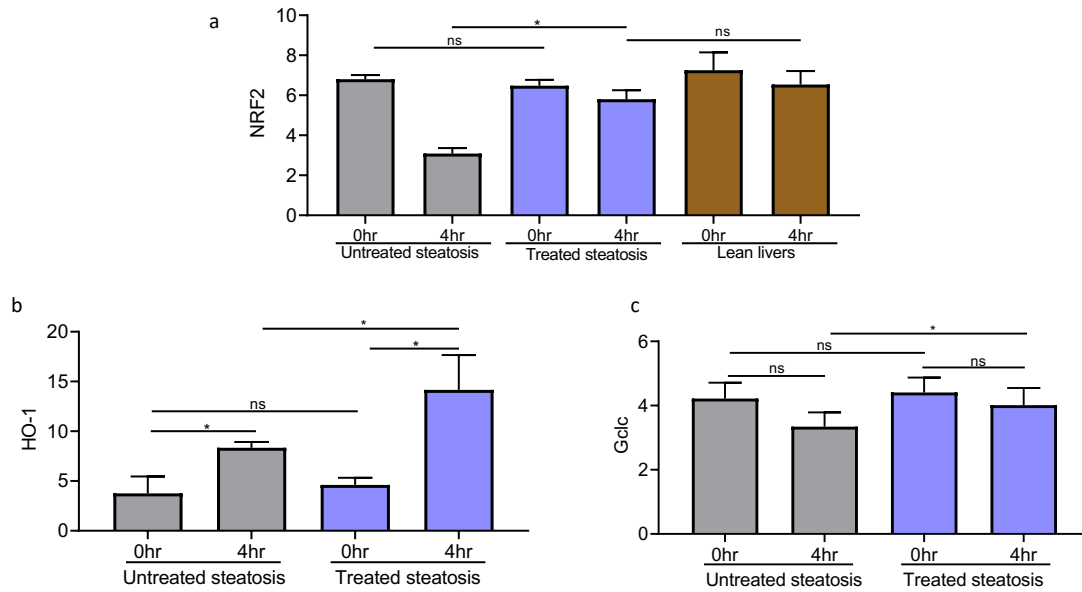


Figure 6. 8 *NRF2 agonist maintains NRF2 expression after NMP in steatotic rat livers.* mRNA expression in fatty rat livers treated with vehicle (control) or NRF2 agonist compared to lean rat livers before and after normothermic machine perfusion (NMP). a) Relative hepatic NRF2 mRNA (to GAPDH) was higher in treated steatotic livers at 4 hours and was comparable to NRF2 levels in lean livers. Treated livers also demonstrated higher b) hemeoxygenase (HO-1) mRNA and c) glutamate-cysteine ligase, catalytic subunit (Gclc) mRNA expression at 4 hours when compared to controls. Data are represented as mean ± SD. *, $p < 0.05$; NS = not statistically significant.

6.4 Oltipraz treatment ameliorated liver injury during NMP

Livers enzymes were measured at 0 and 4 hours using perfusate samples. In both treated and untreated fatty livers, a significant increase in both AST and ALT was observed. In untreated steatotic liver samples, mean perfusate AST was 102 ± 31.7 mIU/g pre-NMP and increased to 1139 ± 406 mIU/g after 4 hours of normothermic preservation ($p = 0.0003$). In NRF2 treated steatotic livers, mean perfusate AST increased from 78 ± 34 mIU/g at 0 hours to 764.4 ± 120.7 mIU/g after 4 hours of NMP ($p < 0.0001$). Perfusate AST levels after NMP were still significantly lower in NRF2 treated steatotic livers when compared to untreated fatty livers ($p = 0.035$) (Figure 6.5a).

In untreated steatotic samples, ALT increased from 253 ± 103.3 mIU/g pre – NMP to 3369.3 ± 960 mIU/g; ($p = 0.04$) after 4 hours of normothermic preservation. In NRF2 treated steatotic livers, mean AST was 103 ± 33.7 mIU/g before NMP and increased to 2841 ± 126.5 mIU/g after 4 hours of NMP ($p = 0.04$) (Figure 6.5b). Despite these increases, treated livers still maintained significantly lower AST values when compared to untreated steatotic livers after 4 hours of NMP ($p = 0.02$).

Both treated and untreated steatotic livers significantly improved lactate clearance during normothermic machine preservation. In the NRF2 treated group, perfusate lactate decreased from 5.2 ± 2 mmol/L to 2.2 ± 0.94 mmol/L ($p = 0.04$). In the untreated steatotic liver group, perfusate lactate pre – NMP was 4.1 ± 0.5 mmol/L and decreased to 1.68 ± 0.28 mmol/L ($p = 0.0001$). Perfusate lactate was comparable in both groups at the 0 hour ($p = 0.34$) and 4 hour ($p = 0.31$) timepoints (Figure 6.5c). Despite a significant decrease in perfusate pH values pre and post NMP in both groups (NRF2 treated livers mean perfusate pH: 0 hr 7.31 ± 0.06 vs 4 hr 6.95 ± 0.37 ; $p = 0.0008$; untreated liver perfusate pH: 0 hr 7.27 ± 0.09 vs 4 hr 7.01 ± 0.09 ; $p = 0.006$) and this remained comparable at the 4 hour timepoint between both treated and untreated fatty livers (Figure 6.5d). Perfusate potassium was significantly lower in NRF2 treated livers at the end of NMP when compared to untreated fatty livers at 4 hours (5.6 mmol/L vs 7.3 mmol/L, $p = 0.0013$).

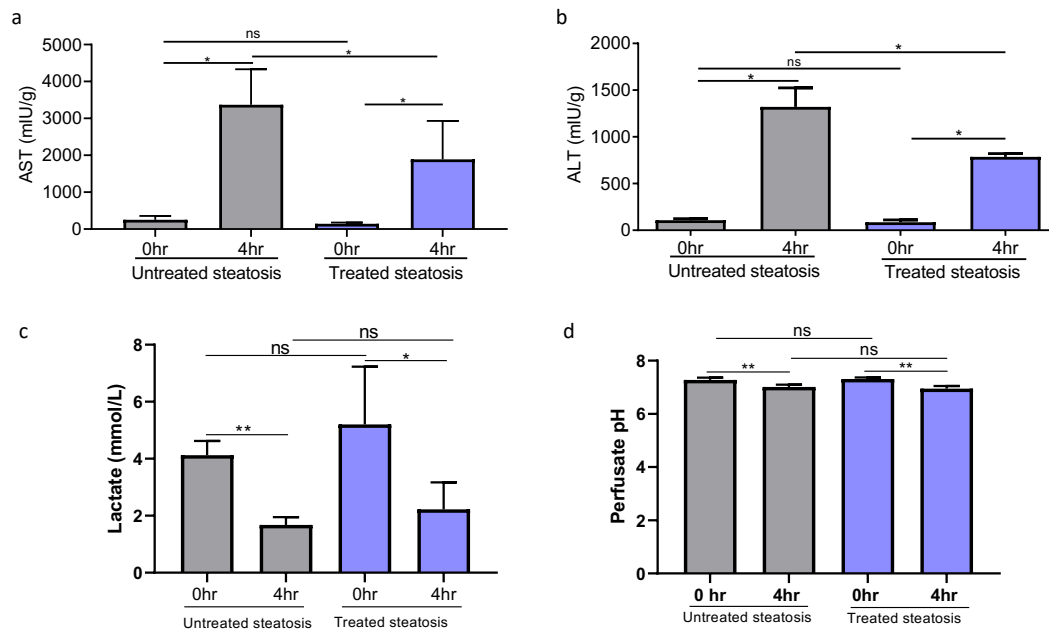


Figure 6. 9 *Biochemical changes to rat fatty liver perfusate during NMP*. Higher NRF2 levels corresponded with lower derangements in **a)** aspartate aminotransferase (AST) and **b)** alanine aminotransferase (ALT). **c)** Lactate improved in both fatty liver groups after NMP and did not differ significantly after 4 hours between groups. **d)** Perfusate pH decreased from baseline (0h) and after 4h normothermic machine perfusion (NMP) in steatotic rat livers untreated or treated with the NRF2 agonist but this was still comparable at the 4 hour timepoint. Data are presented as mean $\pm$ SD. *, p<0.05, **, p<0.01 by t-test.

Histological analysis of treated and untreated steatotic fatty livers did not detect any major inflammatory differences between both groups after 4 hours of normothermic preservation (Table 6.1). Cell vacuolation scores were observed to be significantly higher in the NRF2 treated group (p = 0.049) after NMP when compared to untreated steatotic livers (Figure 6.6).

Table 6. 1 Histological variables relating to pharmacologic NRF2 augmentation in steatotic rat livers[282].

Variable	Control	Treatment	P value
Necrosis	0.5	0.5	0.5
Bile duct inflammation	1.5	1.5	0.352
Vascular inflammation			
Hepatic artery	1	1	0.268
Hepatic vein	1.3	0.5	0.104
Portal vein	1	1	0.268
Vacuolization	1	2.5	0.049
Peri-portal inflammation	1.5	1.5	0.268

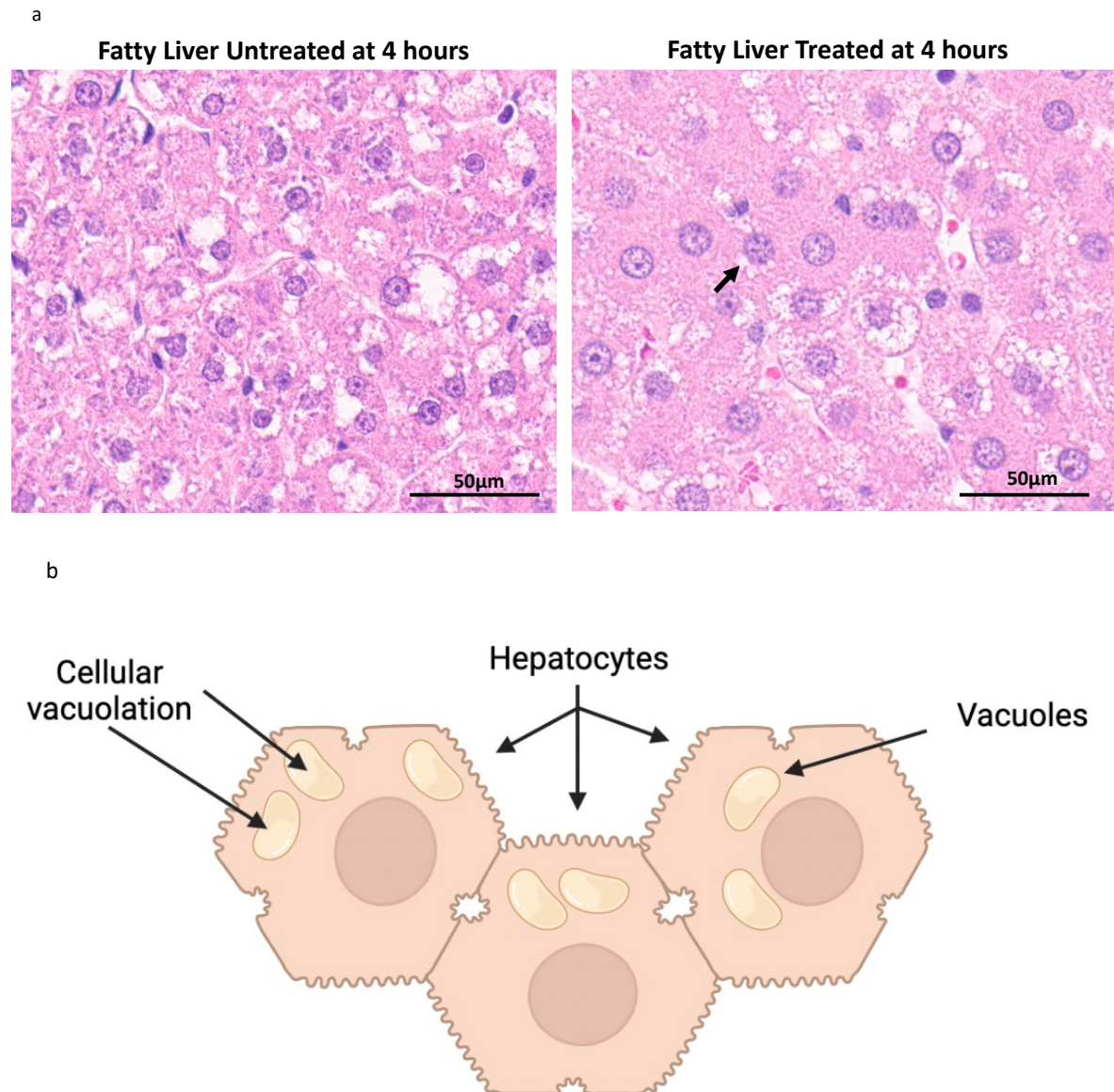


Figure 6. 10 *Representative images of hematoxylin and eosin staining of steatotic rat livers treated with NRF2 agonist or vehicle control following 4 hours of normothermic machine perfusion. a) Increased hepatocyte vacuolation (arrow) was observed in NRF2 agonist-treated livers after NMP[282]. b) Schematic image of hepatocellular cytoplasmic vacuolation, a morphological phenomenon in which cells develop vacuoles during injury.*

6.5 Discussion

Ischaemia causes significant injury to hepatic tissue, however, accruing evidence suggests that the reperfusion phase of IRI is when the damage is most severe [418]. Since steatotic allografts are more susceptible to IRI and are associated with early allograft dysfunction, it seems reasonable to target this aspect of liver transplantation and potentially salvage more livers for transplantation.

Two distinct phases of liver reperfusion injury have been recognised [418]. The early phase occurs during the initial 2 hours of reperfusion and appears to be heavily regulated by Kupffer cell activity [419]. Jaeschke et al studied an in vivo rat IRI model and reported that oxidative stress, which they measure using plasma glutathione disulfide concentrations, peaked at 2 hours of reperfusion during which neutrophil activity increased 3 – 10 fold. The authors also reported Kupffer cell inactivation led to significant ROS suppression, concluding that Kupffer cells are the most dominant source of ROS during the initial stages of reperfusion injury. Additionally, increased recruitment and adherence of CD4+ T lymphocytes to hepatic sinusoids have also been shown to occur in the early phases of reperfusion [420, 421]. Lymphocytes further increase Kupffer cell activation and a recurring cascade of ROS stimulation ensues [418]. The late phase of reperfusion is dominated by ROS and protease release by neutrophils which continues the sequence of parenchymal destruction (Figure 6.7) [420, 422]. Based on this evidence, the current rodent 4-hour NMP model focused on enhancing hepatic function during the early and/or initial late phase of reperfusion and even on a small scale animal model, the observed results are encouraging.

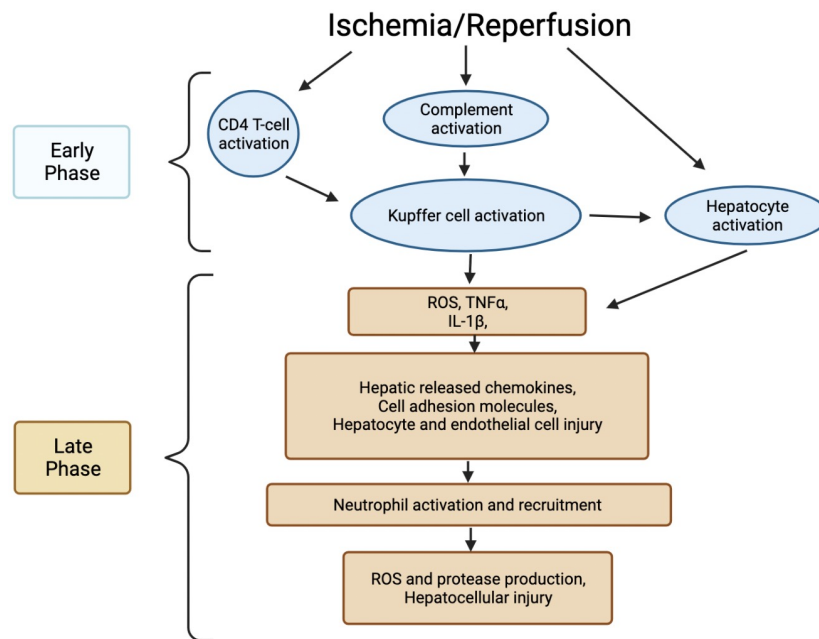


Figure 6. 11 *Schematic illustration of the early and late phases of reperfusion.* Modified from Glantzounis et al *The contemporary role of antioxidant therapy in attenuating liver ischaemia-reperfusion injury: A review* [418]

We used an NRF2 agonist, Oltipraz, as a supplementary adjunct to the NMP process. While previous studies have demonstrated the benefits of NRF2 activators in various IRI models and in a novel HMP system, none have been reported in the setting of NMP [333, 342]. Since IRI incorporates a cascade of several inflammatory players, preservation solutions and ex vivo preservation models create an ideal opportunity to target various pathways and determine their clinical utility through various viability tests. Previous research has demonstrated the ability of Oltipraz to induce the expression of multi-drug resistance-associated proteins (Mrps) through coordinated NRF2 activation [423]. The role of Mrps is in their ability to remove xenobiotics, metabolites and various endogenous substrates that can accumulate in tissues and cause toxicity, important in normal cellular homeostasis. The data thus supports the function of NRF2 in maintaining redox equilibrium and the pharmacological utility of Oltipraz to sustain these processes. Adding to this body of work was a study by Lida et al in a mouse model where they confirmed the ability of Oltipraz in enhancing the detoxification of the urinary bladder-specific carcinogen N-nitrosobutyl(4-

hydroxybutyl)amine (BBN) in an NRF2 dependant manner [424]. Anecdotally, Oltipraz has also been proposed as a possible treatment option in cholestatic liver disease because of the ability of NRF2 to activate bile salt reflux pumps that are primarily expressed in the liver [425]. There are few studies showing its therapeutic potential in hepatic IRI and none in its role in marginal liver allografts. Qian and colleagues used Oltipraz in warm and cold mouse IRI models and demonstrated inflammatory gene repression and reduced inflammatory responses in the experimental group [426]. In the current set of experiments, Oltipraz appeared to work at 3 separate levels; first, increased nuclear translocation of NRF2 was observed on immunofluorescent staining, second, NRF2 levels quantitatively increased on PCR analysis and third, downstream antioxidant genes were also activated (Figure 6.8). These findings support previous work on the mechanistic potential [414] of Oltipraz in promoting nuclear translocation [427], liberating NRF2 from Keap 1 [428] and increasing NRF2 promotor activity [429].

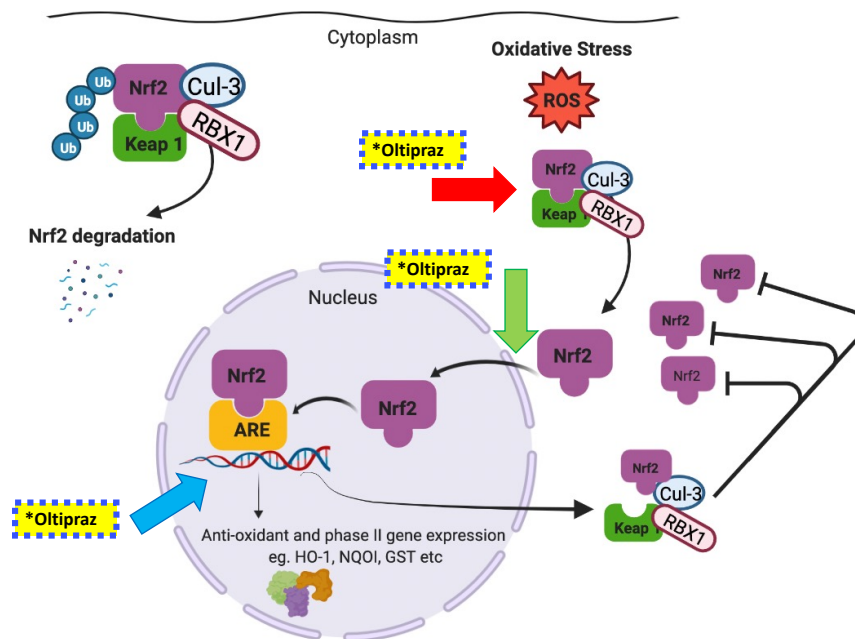


Figure 6. 12 *Pharmacokinetics of Oltipraz*. The current findings support the ability of Oltipraz to release NRF2 from the inhibitory Keap – 1 bind (red arrow), facilitate the nuclear translocation of NRF2 (green arrow), increase its ARE promoter activity and ultimately the expression of downstream antioxidant genes (blue arrow).

Similar to the human livers experiments in the previous chapters, steatotic rat livers decreased NRF2 expression after NMP. Encouragingly, the current experiments demonstrated that Oltipraz could restore replenished NRF2 levels in steatotic livers and re-instate them to normal lean liver levels. The ability of Oltipraz to work on steatotic samples is welcomed progress since these livers account for a considerable proportion of the current donor pool. Additionally, studies have determined that Oltipraz reaches peak concentration at 2 – 4 hours, making it pharmacokinetically ideal in NMP models that are usually applied for 4 – 6 hours [430]. In an NMP setting, Oltipraz can be administered, allowed to function and viability liver testing can then be performed all within the preservation timeframe.

Although IRIs are unlikely to be completely prevented, the effects of this process can potentially be minimised, as is the goal of NMP. Remarkably, Oltipraz treated fatty livers showed significantly lower hepatic enzymes at the end of NMP. Previous studies have shown that NMP can minimise hepatic injury in marginal livers and make them suitable for transplantation [330, 331]. Thus the inherent benefits of NMP cannot be disregarded, however, the added bonus here is NRF2 supplementation. Treated steatotic livers still observed LFT derangements but these were significantly lower than the untreated controls. Supporting this hypothesis are the lower perfusate potassium levels. Both groups demonstrated relatively high levels of potassium and this is likely related to the rotary pumps on the NMP apparatus and laminar flow dynamics causing haemolysis [431]. This appears to be an inherent weakness in the design of our NMP apparatus and may need to be optimized. Despite this limitation, Oltipraz treated livers still had significantly lower potassium levels in the tested perfusate. This could be related to previous work by Shih and colleagues which demonstrated that erythrocyte haemolysis was induced by free radicals and that this phenomenon was due to oxidative stress and simultaneously regulated by NRF2 antioxidant defences [291]. This could further support the use of NRF2 agonists in marginal liver NMP systems.

The histological findings were less remarkable than previously demonstrated on the human allograft experiments. This could, once more, be an inherent weakness of our rat NMP device or also relate to the short duration of NMP in the current experiments. A notable

finding in the current results was the increased vacuolization in treated steatotic livers. A possible explanation for this observation could be that these vacuolated hepatocytes are cells adaptively altering their phenotype to resist injury rather than undergoing degeneration, a finding that has been previously investigated in hepatocellular injury [432]. This outcome did not result in increased necrosis, and interestingly, other markers of injury were lower in NRF2-treated livers. It has also been postulated that vacuolation in cells could be a physiological response to ultimately “limit damage”, however little is known about the homeostatic mechanisms regulating these changes [433]. If damage limitation fails, cells may die quickly in an uncontrolled manner further driving widespread injury and oncotic necrosis or “cell explosions” which coincidentally causes a dramatic rise in serum transaminases [11].

6.6 Implications

What is already known:

Marginal liver allografts are currently being rescued from discard by pre conditioning and restoring them using novel normothermic preservation techniques. Steatotic allografts are a leading cause for organ discard and there is convincing evidence that these allografts are more susceptible to IRI and are predisposed to early allograft dysfunction. A key strategy to optimise the results of steatotic livers is to utilise novel preservation concepts and pharmacological additives to attenuate injury and mediate hepatoprotective effects. Some studies have suggested that NRF2 activation may alleviate IRI in livers and this appears to be a potential therapeutic target.

What is new:

NRF2 was modulated in steatotic livers and, by using an NRF2 agonist, we have demonstrated the ability to restore NRF2 levels in fatty livers during NMP to reach normal lean liver levels. In doing so, we have also demonstrated an increase in antioxidant gene expression and these factors were associated with significant improvements in hepatic enzyme derangements during NMP. NRF2 modulation may also regulate physiological cell death by promoting cellular vacuolation in a controlled manner without increasing widespread necrosis. The data also suggests that not all steatotic livers are incapable of generating an NRF2 response and that these livers can also experience less injury during NMP making them suitable for transplantation.

Potential impact on future practice:

Novel NMP technology may offer a therapeutic window to regulate the early phase of reperfusion injuries. Supplementing steatotic allografts, which are known to be NRF2 deficient, with hepatoprotective components during NMP could revitalize suboptimal allografts and expand the donor pool. The ability of some livers to react positively by increasing NRF2 after NMP could help determine which livers are likely to benefit from the machine and ultimately reduce unnecessary liver discard.

Chapter 7

General discussion and conclusions

7.1 Inflammatory and homeostatic processes within the liver

7.1.1 Immunological disruptions in the hepatic microenvironment

The balance between key immunoregulatory pathways is central to the inflammatory and homeostatic processes occurring within the liver. The absence or dysregulation of effective immune pathways in response to injury underpin the main molecular mechanisms of tissue damage that are seen in a diverse array of liver diseases. The differential expression of certain physiological protein molecules, which aim to restore the repair capabilities of a damaged organ, may also be compounded by a challenging hepatic metabolic profile. Accordingly, elucidating the immunological basis of these pathways would allow better tailoring of medical protocols and help identify novel targets for therapeutic interventions.

Liver disease is responsible for approximately 2 million deaths globally per year [32]. In the US alone, over 50,000 patients died from chronic liver disease or cirrhosis, an astronomical rise from the preceding years [434]. In the UK, liver disease has now become one of the leading causes of premature death accounting for more than 62,000 years of working life loss each year [435]. As noted repeatedly throughout this thesis, liver transplantation is the only definite treatment option for patients with end stage liver failure. Modern benchmarks include an overall and graft survival both exceeding 90% and 70% at 1- and 5 -years, making liver transplantation a well-accepted curative alternative for select patients [51]. Increased demands have outweighed supply and between 20% and 30% of patients on the liver waiting list will die before they receive a transplant [38, 39, 436]. In an effort to respond to organ shortages, the international consortia has had to devise innovative surgical techniques such as split or partial liver transplants and living donors while also considering changing patient population demographics which are now giving rise to extended criteria or marginal liver allografts [41]. The exact definitions of a marginal donor remain controversial and individual transplant units appear to use their own policies based on generic national guidelines to make decisions on allograft acceptance or discard [46]. Encouragingly, recent evidence has suggested that some previously declined livers are still suitable for clinical use and this has been supported by novel ex vivo allograft preservation technology [330, 331]. Predicting how livers will respond to normothermic reconditioning and deciding which livers should be used is a laudable goal. The constant challenge in marginal organs is the

multistage IRI process to which they are more susceptible to, limiting their use. Collectively, the current set of experiments raise the possibility that stratifying marginal allografts based on anti-oxidant modulation may help identify which livers could be realistically rescued for transplantation.

Targeting the IRI process holds promise in optimizing liver function and enhancing cytoprotective strategies, making livers safer to use. The liver is an immunologically complex organ hosting an extensive immune cell repertoire within a tightly regulated homeostatic microenvironment. As such, immunological tolerance is balanced by a pro- and anti-inflammatory milieu resulting in deleterious consequences if signalling systems proceed unchecked. This is the scenario in oxidative stress and excessive ROS production leading to an extreme hepatic inflammatory response during IRI through the oxidation of important cellular constituents and signalling proteins. In physiological states, ROS production play a key role in cellular defence and protection from both exogenous and endogenous threats by acting as signalling molecules generating the production of several proinflammatory cytokines. Consequentially, when ROS production is unchecked, an overwhelming inflammatory response results in deleterious collateral damage.

7.1.2 The tolerogenicity of marginal allografts

Increased life expectancy estimates and the increasing prevalence of metabolic conditions in the general population over the last 3 decades are thought to be causation factors for increasing liver discard [37]. Potential donors are now older, have higher rates of obesity and diabetes and a greater prevalence of liver disease [84, 401]. Demographic population shifts have also impacted the donor pool forcing units to consider the use of marginal livers which historical research had previously linked to poor post-operative outcomes [404, 437, 438]. A previous study carried out from this academic unit demonstrated differential expressions of NRF2 in allografts from older donors which had worse clinical outcomes during surgery [247]. This added to a growing body of work at the time attempting to optimise allograft selection and naturally favoured younger liver donors. Since then, a shifting patient demographic profile with increased metabolic conditions and longer life expectancy rates have categorically hampered the quality of liver allografts as we are seeing more ECD livers from older donors with underlying liver disease. Thus, understanding the

molecular biology of marginal livers and their antioxidant potential may help uncover new therapeutic targets. In chapter 3, we demonstrated the wide variability in NRF2 expression within a sample of discarded liver allografts that were declined for transplant due to multiple quality concerns and this reflected the clinical reality of organ allocation sequences. It has become increasingly clear that genetic variation exists among individuals and may account for interindividual gene expression, immune responses and allograft susceptibility to injury [439]. This variation may have resulted from absent NRF2 protein reserves or physiologic decline in some discarded allografts exacerbating parenchymal injury [33, 34]. Donors of livers with high NRF2 expression demonstrated significantly lower pre-operative liver enzymes. Moreover, our experiments have confirmed the expression of NRF2 in both parenchymal and non-parenchymal cells and increased CD3+ cell infiltration was noted in livers with lower expression of NRF2 which ultimately demonstrated a poorer histological architecture. Given that lower expression levels of NRF2 was associated with significant injury, the data implied that some discarded livers lacked inherent cytoprotective mechanisms which could influence their behaviour during the transplantation process. We acknowledge that there are some limitations to the findings presented in this thesis. We have studied a small number of discarded liver allografts with varied ischaemia times. This likely disrupts a multitude of cellular functions, but reflects the reality of clinical practice and variety of procured liver allografts available. Additionally, the predominance of male donors in the current discarded liver pool, although consistent with real life practice, statistically challenges efforts to investigate sex differences in NRF2 expression which have been previously noted [440-442]

The prevalence of steatosis, defined as the abnormal accumulation of fat (>5%) in hepatocytes, in the studied human liver allografts was 45% of samples and this aligns with current population based studies identifying steatosis as a leading cause for organ discard [401]. The indolent nature of fatty liver disease is an additional concern and studies have suggested that this could have wide implications on transplant services nationally [84]. We demonstrated that livers with steatosis exceeding 40% appear to have much lower expression levels of NRF2 and this could explain the increased susceptibility of severely steatotic livers to IRI. The observed metabolic differences between high NRF2 expression livers and low NRF2 expression livers were also notable. High NRF2 environments demonstrated

significantly higher expression levels of pAMPK compared to low NRF2 livers. This pathway is recognised as a potent intracellular sensor regulating glucose and lipid metabolism with the ability to reduce ATP consuming pathways in times of cellular energy depletion [313, 315, 320]. The findings support the role of NRF2 in maintaining functional hepatic processes, although the direct associations still warrant further exploration. Drawing some similarities to these correlations, the increased pERK levels in livers with a high expression level of NRF2 is also significant. The ERK pathway plays a central role governing cell proliferation, differentiation and survival [443]. A seeming paradox exists when ERK phosphorylation and subsequent activation occurs with reports of cell growth induction following rapid transient activation of ERK while a large and sustained response appears to suppress cell proliferation [444-446]. Based on the current results, it could be argued that high NRF2 expression in discarded livers may also relate to the proliferative and regenerative potential of allografts, an important concept considering the encountered physiologic stress during surgery. A key stage during the implantation process is an allograft's ability to restore function following a period of ischaemia and regenerate accordingly in the presence of ROS influx during the reperfusion phase. Characterizing which allografts are best at achieving this naturally and which organs may benefit from therapeutic intervention prior to transplantation could minimize excessive discard.

7.1.3 NRF2 and hepatic resistance to injury

In chapter 4, we hypothesised that livers with higher expression levels of NRF2 protein would perform better in a simulated IRI model using a lab-based NMP device. The role of ex vivo normothermic machine preservation for marginal livers is a dominant theme in current clinical research and evidence suggests that many potential donor organs are underutilised. The underlying molecular mechanisms responsible for marginal liver injury remain poorly characterised. Determining the suitability of a graft for transplantation based on objective antioxidant features rather than subjective surgeon assessments could be an important achievement. We reported a positive association between high NRF2 expression and allograft responses to NMP. Livers with higher hepatic NRF2 expression consistently demonstrated lower LFT derangements following machine perfusion and also maintained immune protection to the vascular endothelium, most notably against significant hepatic artery inflammation. In this regard, NRF2 expression in liver allografts may not only denote

improved liver quality, but its upregulation following NMP can be viewed optimistically by protecting both parenchymal and vascular structures and increasing resistance to IRI. NMP also appeared to suppress Keap-1 expression which may further drive NRF2 activation and combat oxidative stress. Existing data suggests that reducing IRI in DCD livers may occur through NRF2 signaling pathways in hypothermic settings [333]. Although both normothermic and hypothermic machine preservation methods reduce acute liver injury, only NMP allows for allograft viability assessments that may guide clinical decision making and organ selection. Our experiments demonstrated that livers with higher expression of NRF2 could clear lactate in a more effective manner and post-NMP values were consistent with current viability criteria accepted in real life clinical scenarios. This result suggested that reductions in oxidative stress through NRF2 signaling may reduce inflammation in marginal livers while also restoring their functional potential. Interestingly, a small sample of livers with moderate to severe steatosis still appear to show variable expression levels of NRF2 and benefit from the NMP techniques. Our results also suggest that 1 in 4 steatotic livers could still meet NRF2 expression requirements, respond positively during preservation and pass viability assessment criteria indicating the heterogeneous pool of discarded organs. Some rejected steatotic livers could be salvageable for clinical use if an NRF2 rich environment is present and these findings can add to current discussions of ECD definitions and efforts to optimize marginal allografts [47, 48]. Once more, it must be acknowledged that a small number of livers using NMP were studied in this chapter. The number of ECD livers allocated for research purposes, that are technically and anatomically suitable for *ex vivo* machine perfusion, is low and using additional livers should be considered to further validate the molecular evidence of NRF2 activation. The cost of each cycle of NMP is also substantial and is reliant on disposable parts, human blood and sterilization equipment that need to be considered in a lab-based setting

7.1.4 Targeting NRF2 to alleviate hepatic injury

Controlling ROS production is the underlying principle of utilising NRF2 activators to attenuate IRI during liver transplantation. It is thought that NRF2 is expressed ubiquitously in several cell lines and modulating redox metabolism by activating NRF2 may attenuate inflammation-related ROS signaling [447]. In chapter 5 we show a diverse range of NRF2 activators used in the context of liver transplantation and encouraging results minimizing

inflammatory injury while still maintaining satisfactory hepatic function. Most activators focused on optimizing the nuclear translocation of NRF2 to stimulate its activity and the release of downstream antioxidant genes within its axis. In all studies, IRI was attenuated through pharmacological activation. The studies were ultimately limited by the predominant use of murine models and that grafts were not implanted into recipient hosts to assess the extent of IRI protection and correlate this with additional functional outcomes. Moreover, the feasibility of NRF2 activation on lean liver allografts was encouraging, albeit a clinical setback in the era of worsening donor allograft quality and marginal donors.

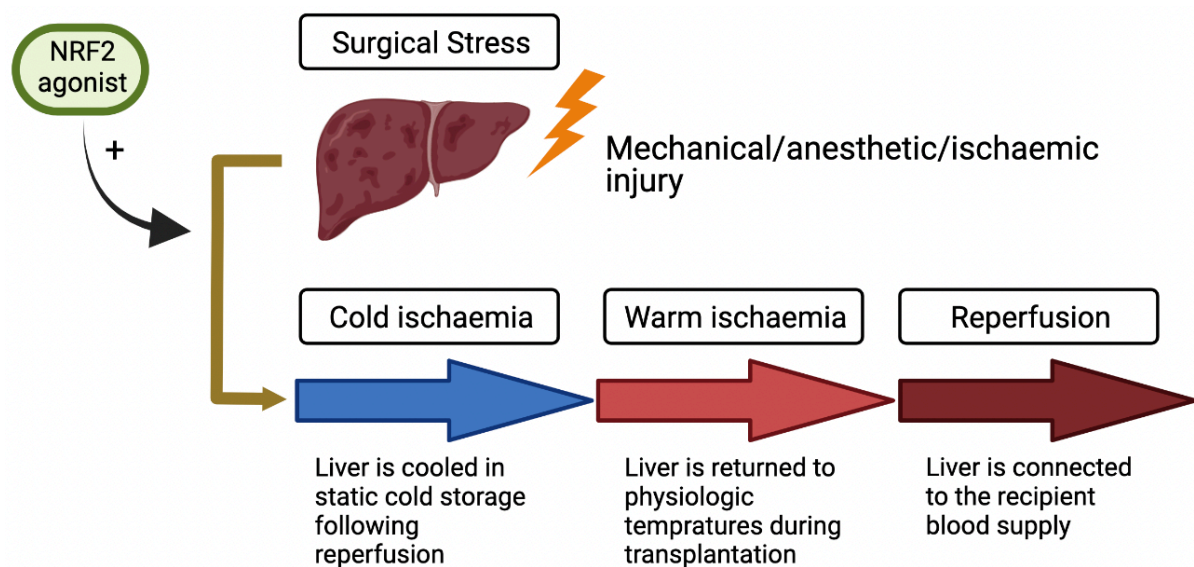


Figure 7. 1 *Proposed clinical utility of NRF2 activation in liver transplantation.* The allograft is exposed to multiple phases of stress initially related to surgery and followed by IRI. NRF2 activation may confer additional hepatic protection and homeostatic restoration. This could be added directly to the allograft prior to cold or normothermic preservation.

Finally in Chapter 6, we explored the probability of NRF2 modulation in marginal liver allografts. The novelty of the current experiments was testing this strategy in a murine fatty liver model using NMP technology. Using Oltipraz, an NRF2 agonist, appeared effective in increasing nuclear translocation of NRF2, cellular expression levels and downstream release of anti-oxidant genes (Figure 6.8). When fat deposition exceeds supraphysiological levels resulting in pathological steatosis, routine β -oxidation is unable to maintain lipid metabolism and, once more, homeostasis is lost. In an effort to restore balance, an increase in free fatty acid β -oxidation is observed, however, this causes increased rates of electron

leakage from the mitochondrial respiratory chain and results in high ROS production. This contributes to a constant state of oxidative stress in hepatosteatosis which may explain eventual loss of physiological reserves in fatty livers. In the current rodent model, NRF2 augmentation reduced allograft injury during NMP and steatotic liver NRF2 reserves were restored to normal lean liver levels. This finding can be viewed optimistically as it implies the feasibility of recovering antioxidant systems in marginal livers. This knowledge may lead to the development of distinct therapeutic targets that could enhance marginal liver function during organ preservation. The findings also suggest that marginal livers could acquire resistance to IRI and fully understanding the mechanisms behind this method of hepatoprotection will allow us to identify predictive biomarkers and synergistic agents that can mitigate injury. The final hurdle would be to transplant treated livers in the animal setting and investigate whether NRF2 protection is maintained *in vivo* as our findings also lack this observation. Despite these limitations and challenges, the study identifies and confirms the feasibility of targeting NRF2 in discarded marginal livers in a controlled setting and its clinical relevance in allograft function.

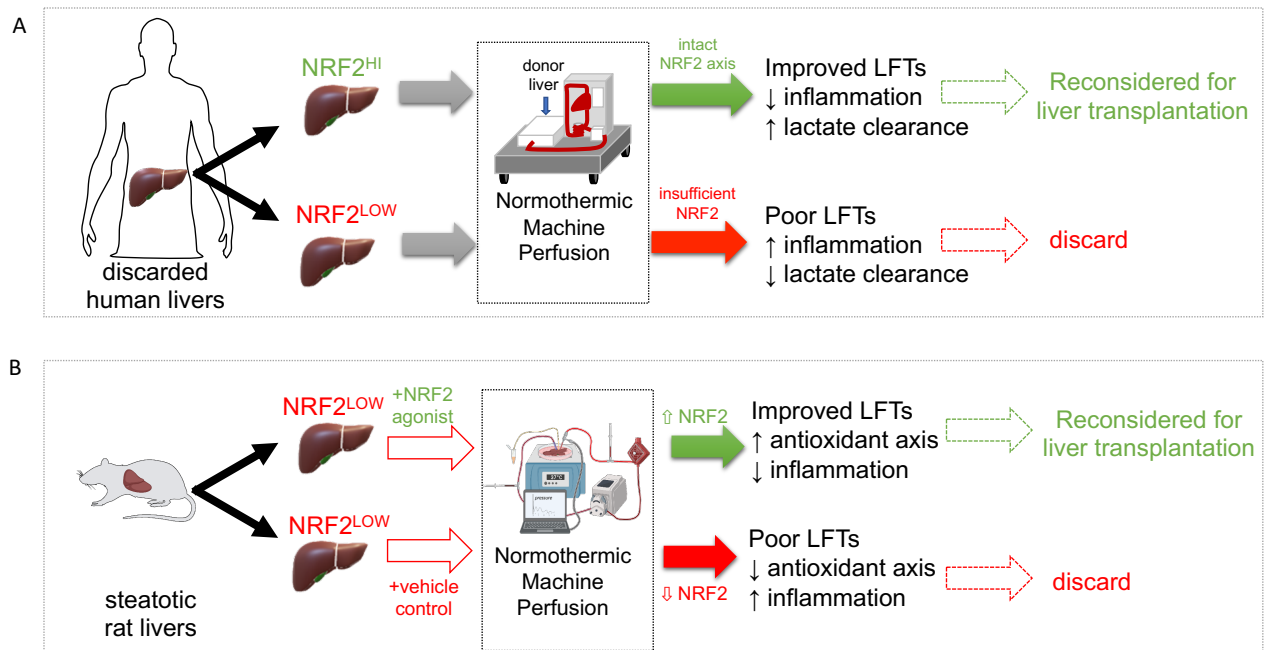


Figure 7. 2 Thesis hypothesis and overview of results

Proposed feedback mechanism.

A. Upper panel: Human livers with varying etiologies are discarded from the liver transplant donor pool. When livers are stratified based on hepatic NRF2 protein expression, livers with high levels of NRF2 (NRF2^{HI}) expression had improved responses to normothermic machine perfusion compared to livers with lower levels (NRF2^{LOW}). Less hepatic injury was observed in NRF2^{HI} livers, and this group represents additional livers which may potentially be salvaged from the discarded organ pool. Future research will determine the suitability of these for transplantation (unfilled green arrow). NRF2^{LOW} livers underwent further injury and remained unsuitable for safe clinical use (unfilled red arrow).

B. Lower panel: Hepatic steatosis is associated with reduced NRF2 levels. When treated with an NRF2 agonist, steatotic rat livers demonstrated evidence of NRF2 recovery and increased levels of downstream antioxidant genes (HO-1, Gclc) following *ex vivo* perfusion (solid green line). The NRF2 agonist also led to improved transaminases in

steatotic rat livers. Future research should analyze the suitability of NRF2 agonist-treated livers for transplantation (unfilled green arrow). Untreated steatotic livers demonstrated a further reduction in NRF2 levels following machine perfusion (solid red arrow) and resulted in higher transaminase levels when compared to the treated group. Such livers would appear unsuitable for transplantation due to this degree of hepatic injury (unfilled red arrow).

7.2 Future directions

This study highlights several elements that could aid efforts in minimizing the current rate of organ discard. Locally, in light of current evidence from Irish data that obesity rates and NAFLD are rising in Ireland [448, 449], the next logical step would be to assess public health trends and determine whether these factors have altered our donor pool. This could help predict and map out future challenges to the Irish healthcare system and serve as a further impetus to design public health interventions and tackle the “silent epidemic”[97].

Further examination into the application of the NRF2 pathway in marginal allografts that are more susceptible to IRI is also warranted and clinically validating its role as a biomarker can only be confirmed by transplantation. Hepatic zonation and the spatial variability of metabolic functions may influence how anti-oxidant defence mechanisms function within marginal liver allografts and technologies using single-cell transcriptomics and RNA sequencing may help map antioxidant identifiers that further implicate IRI in marginal liver transplantation. Moreover, assessing which cells can withstand anaerobic conditions and resist oxidative injury the longest may help determine the full capability of the NRF2 pathway. Individual genetic variations may explain why some allografts are more susceptible to injury and the role of genetic markers of resistance requires further clarification. Identifying specific genetic weakness may improve molecular targeting. Similar data in the context of hepatic fibrosis may also forward discussions on oxidative stress in this setting, informing ongoing discussions on the reversibility of this disease and the restoration of hepatic function.

Allograft prognostication has also been a clinical challenge in liver transplantation and is more complex when marginal livers are being considered. While NMP technology has the potential to improve organ utilization and patient outcomes, this is still associated with increased cost. Determining which livers are likely to derive a meaningful benefit from NMP based on their molecular profile may help advance allograft selection for NMP, and this may be answered, once more, by genetic analyses. In real life clinical scenarios, using ELISA test kits may align with the urgency of decision making for allograft acceptance and help quantify NRF2 and other relevant biomarkers within perfusate samples. Determining a suitable “cut-off” value for anti-oxidant protein expression would be invaluable. As delineated in this thesis, discarded allografts are not a homogenous sample and some appear to functionally perform better than others. The difficulty with this concept is that in order to identify an accurate relative cut-off for NRF2 expression, a complex series of mathematical modelling using a substantial number of both normal and abnormal allografts will have to determine this. This is further challenged by the widespread genetic variation within a heterogenous donor population and it is clear that some livers can restore antioxidant function why a proportion remain resistant to regeneration. Translating these findings to all solid organ transplant is a compelling goal. Some of the favourable changes observed in livers in the context of NRF2 would be equally valuable in heart, lung and kidney allografts. How the NRF2 pathway may interact with various organs is a complex question and studies measuring these associations may help determine which biomarkers are most relevant. It is the hope of this work that by understanding antioxidant mechanisms and how they are induced, we can rationally tailor treatment to individual marginal allografts, fulfilling the prophecy of personalised medicine.

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Publications



REVIEW ARTICLE OPEN

Inflammatory processes in the liver: divergent roles in homeostasis and pathology

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The hepatic immune system is designed to tolerate diverse harmless foreign moieties to maintain homeostasis in the healthy liver. Constant priming and regulation ensure that appropriate immune activation occurs when challenged by pathogens and tissue damage. Failure to accurately discriminate, regulate, or effectively resolve inflammation offsets this balance, jeopardizing overall tissue health resulting from an either overly tolerant or an overactive inflammatory response. Compelling scientific and clinical evidence links dysregulated hepatic immune and inflammatory responses upon sterile injury to several pathological conditions in the liver, particularly nonalcoholic steatohepatitis and ischemia-reperfusion injury. Murine and human studies have described interactions between diverse immune repertoires and nonhematopoietic cell populations in both physiological and pathological activities in the liver, although the molecular mechanisms driving these associations are not clearly understood. Here, we review the dynamic roles of inflammatory mediators in responses to sterile injury in the context of homeostasis and disease, the clinical implications of dysregulated hepatic immune activity and therapeutic developments to regulate liver-specific immunity.

Keywords: homeostasis; immunology; inflammation; liver*Cellular & Molecular Immunology* (2021) 18:1375–1386; <https://doi.org/10.1038/s41423-021-00639-2>

AN INTRODUCTION TO THE IMMUNOLOGICAL STRUCTURE OF THE LIVER

The liver is an immunologically complex organ that functions as a physiological bridge between gut-derived molecules and the systemic circulation. Approximately 80% of the hepatic vascular supply is provided by the portal venous system, which results in a low-pressure blood system with large amounts of bacterial products and other foreign molecules that are presented to the immune system. Consequently, the liver must tolerate these potentially immunogenic or inflammatory foreign molecules while maintaining constant immunosurveillance for infectious pathogens and liver malignancies. The hepatic microstructure is composed of an extensive repertoire of immune cells embedded in a meshwork of liver sinusoids through which hepatic venous blood passes and mixes with oxygen-rich arterial blood from the hepatic artery. These sinusoids are lined by specialized liver sinusoidal endothelial cells (LSECs) that contain numerous fenestrations, allowing molecules to freely diffuse through to the underlying hepatocytes, which perform the central metabolic functions of the liver¹ (Fig. 1). This alignment facilitates the rapid exchange of blood-borne molecules and metabolic processing in the liver, as well as extensive interactions with local and systemic immune populations.

Hepatic homeostasis relies on effective regulation of the intimate interactions between resident and infiltrating immune cells and nonhematopoietic hepatic cells. Resident immune cells populate the liver sinusoids and the subendothelial compartment known as the space of Disse, which is located between hepatocytes and LSECs; these immune cells are a diverse

population, including professional antigen-presenting cells (APCs), myeloid cells, and specialized innate and adaptive lymphoid cell populations.¹ Many hepatic immune populations play vital roles in homeostasis, tissue repair, maintaining immune tolerance, and modulating liver inflammation. Here, we review the dynamic roles of hepatic inflammatory immune responses in homeostasis and how inflammatory responses to sterile injury lead to pathology, specifically in the context of nonalcoholic steatohepatitis (NASH) and ischemia-reperfusion injury (IRI).

HEPATIC IMMUNE CELL REPERTOIRES

Hepatic immune cell populations play diverse roles in regulating inflammation and immune responses. Kupffer cells (KCs), which account for 80–90% of the total population of fixed tissue macrophages and almost one-third of the nonparenchymal cell population in the liver, are key.² These liver-specific myeloid cells play critical roles in the recognition of blood-borne pathogens and the clearance of invading microbes.^{3,4} These cells have key roles in identifying, phagocytosing, and eliminating “foreign” or “dangerous” antigens via PPRs, complement receptors, and Fc receptors. Consistent with their macrophage-like functions, KCs exhibit increased phagocytic activity; however, in contrast to other myeloid populations, KCs produce proinflammatory and anti-inflammatory factors, illustrating their dichotomous role in tolerogenic defense and inflammation.⁵

Dendritic cells (DCs) are also important inducers of tolerance in the liver, although these cells are also capable of antigen presentation and type 1 interferon production.^{6–8} DCs are

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Received: 17 November 2020 Accepted: 10 January 2021

Published online: 16 April 2021

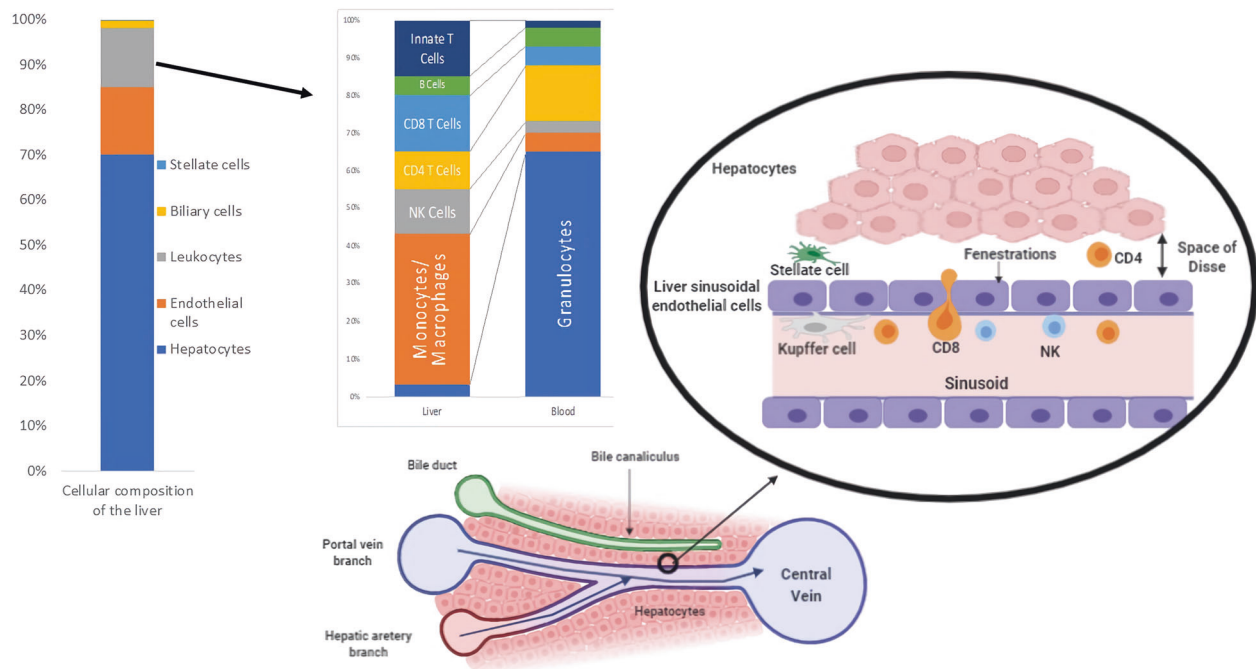


Fig. 1 The cellular composition of the liver. Venous blood from the gut mixes with oxygenated arterial blood, drains through the sinusoids and passes through plates of hepatocytes to the central vein. Hepatocytes secrete bile into the canaliculi, and these secretions ultimately flow into the bile ducts. The sinusoids are lined by specialized, fenestrated liver sinusoidal endothelial cells that allow blood to pass through the space of Dissé to the underlying hepatocytes. Within the blood, Kupffer cells adhere to the endothelial wall, while resident lymphoid and myeloid immune cell populations are found around the portal tract and throughout the parenchyma

categorized into myeloid and plasmacytoid subtypes and are primarily located around the central veins and portal tracts.⁹ Under physiological conditions, DCs are phenotypically immature and tolerogenic and are thought to play an important role in tolerogenicity posttransplantation;¹⁰ however, proinflammatory DCs develop in response to liver injury, stimulating cytokine secretion and T cell responses.¹¹ There is evidence of DC participation in anti-inflammatory activities, particularly in the context of IRI, indicating their dual immunosuppressive and proinflammatory roles.¹²

Classic neutrophils are rarely detected in the healthy liver. However, neutrophil subsets with immunoregulatory properties, also referred to as type 2 neutrophils (N2), have been found among tumor-associated neutrophils (TANs) in hepatocellular carcinoma.¹³ These neutrophil subsets share phenotypic and functional features with mature low-density neutrophils or granulocyte-like myeloid-derived suppressor cells (MDSCs),¹⁴ which are known to suppress T-cell activation and, in the context of cancer, promote cancer growth.^{15,16} MDSC expansion has also been described in nonalcoholic fatty liver disease (NAFLD).¹⁷ The immunosuppressive activity of N2/TANs/G-MDSCs has been associated with the upregulation of various factors that promote an immunosuppressive environment, such as arginase or indoleamine 2,3-dioxygenase.¹⁸

Classic T lymphocytes account for up to 50% of lymphocytes in the liver, and B cells make up only 5% of the hepatic lymphocyte population.¹⁹ While CD8⁺ T cells usually outnumber CD4⁺ T cells at a two-to-one ratio in the liver, both cell types have activated phenotypes. A large proportion of liver T lymphocytes have innate-like features.²⁰ These T cells coexpress CD56 and/or CD161 and include NK T (NKT) cells, mucosal-associated invariant T cells, and $\gamma\delta$ T cells, which are involved in hepatic immunity and tissue remodeling.^{21–23}

Innate B cells that express high levels of CD5 are also found in the human liver and are likely to be immunoregulatory, although

it is unknown whether these cells produce the same suppressive cytokine milieu as the systemic subtype.²⁴ Regulatory T cell-induced Breg cell formation in a mouse model of hepatic autoimmunity requires IL-21 but neither IL-10 nor TGF- β .²⁵ Human studies have consistently demonstrated B-cell expansion in hepatitis C-infected liver tissue, and these cells potentially contribute to the suppressive immune environment that is typically observed in chronic HCV-infected livers.^{24,26–28}

In humans, NK cells account for 30–50% of the hepatic lymphocyte population,²⁹ while in mice, only 5–10% of hepatic lymphocytes are NK cells.^{5,30} These cells have important antiviral and antitumor activities and can also interact directly or indirectly with liver APCs, KCs, and DCs to regulate hepatic immune responses.³¹

Several studies using murine and other animal models have defined the role of the liver in hematopoiesis.^{32–34} Mouse studies have demonstrated that in the absence of a spleen, extramedullary hematopoiesis can occur in the liver.³⁵ In adult mice, the lymphopoietic capacity of adult liver cells was similar to that of bone marrow transplantation, suggesting a key role in liver transplantation tolerance and T-cell reconstitution in recipients.³⁶ Most human hematopoietic stem cell (HSC) studies are centered around fetal liver development, although these stem cells are also present in the adult liver.³⁴ Lymphoid progenitors (LPs) have also been detected in healthy liver tissue,³³ and LPs that express CD56 are more common in the liver than in the bone marrow.³⁷ The expansion of the HSC pool that expresses markers of myeloid differentiation has been described in liver metastasis³⁸ and may contribute to the increased numbers of granulocytic cells or MDSCs observed in metastatic livers.

Within the liver, nonhematopoietic cell populations also possess important immune functions.⁵ LSECs are involved in hepatic leukocyte recruitment via Toll-like receptor 4 (TLR4) activation and appear to exhibit both tolerogenic and proinflammatory functions through CD4⁺ and CD8⁺ interactions. Antigen-presenting

Table 1. The expanding repertoire of intrahepatic immune cell populations identified by single-cell RNA sequencing

Immune cell compartment	Subpopulations identified	Reference
Kupffer cells (CD163 ⁺ VSIG4 ⁺)	LIRB5 ⁺ CD5L ⁺ MARCO ⁺ HMOX1 ^{high} immunoregulatory subset CD1C ⁺ FCER1A ⁺ antigen-presenting subset	Aizarani et al. ⁴⁶
Kupffer cells (CD68 ⁺)	LYZ ⁺ CSTA ⁺ CD74 ⁺ inflammatory macrophages CD5L ⁺ MARCO ⁺ VSIG4 ⁺ LILRB5 ⁺ HMOX1 ⁺ tolerogenic macrophages	MacParland et al. ⁴²
Mononuclear phagocytes	CD163 ⁺ MARCO ⁺ TIMD4 ^{+/−} Kupffer cells TREM2 ⁺ CD9 ⁺ scar-associated macrophages	Ramachandran et al. ^{47,48}
Dendritic cells	CLEC9A ⁺ XCR1 ⁺ CADM1 ⁺ cDC1 CD1C ⁺ FCER1A ⁺ CLEC10A ⁺ cDC2 LAMP3 ⁺ CD80 ⁺ CD83 ⁺ CCR7 ⁺ activated migratory DCs	Zhang et al. ¹³⁴
Natural killer cells	CD160 ⁺ IL2RB ⁺ CXCR6 ⁺ tissue-resident NK cells CX3CR1 ⁺ GNLY ⁺ GZMB ⁺ classic NK cells HMBG2 ⁺ MK67 ⁺ cycling NK cells	Zhao et al. ⁴⁹

capabilities have also been described in hepatocytes.³⁹ In response to hepatocyte antigen presentation, an effector CD8⁺ T-cell response is initiated when the initial antigen load is low, in contrast to the functionally exhausted population of CD8⁺ cells with PD-1 expression that is observed during abundant hepatic antigen presentation.^{22,40} Furthermore, hepatic injury and inflammation can result in IL-10 induction by CD4⁺ T cells as a consequence of hepatocyte activation to restore immunological homeostasis and regulate a potentially overwhelming immune response.⁴¹

Identification and characterization of immune cell populations by single-cell RNA sequencing analyses

The spectrum of resident hepatic immune cell populations is currently expanding through the use of single-cell RNA sequencing approaches.^{42,43} This technology has allowed for the intimate analysis of a comprehensive immune cell population and has identified a number of novel intrahepatic immune subsets (Table 1). The challenge in applying these approaches to liver tissue relates to the spatially graded hepatic microenvironment and zonation patterns, although new technologies are beginning to include spatial localization data.^{44,45} The loss of fragile liver cells during sample processing and the consequent underrepresentation of non-parenchymal cell populations in subsequent single-cell RNA sequencing datasets is another limitation of these technologies.⁴²

A number of liver single-cell RNA sequencing studies have focused on heterogeneity within the KC population. These studies have identified two subpopulations in the human liver: a proinflammatory antigen-presenting KC subset and a tolerogenic KC subset (Table 1).^{42,46} There is also a distinct scar-associated macrophage subpopulation present in patients with liver cirrhosis,^{47,48} highlighting the importance of comparative analysis of healthy and diseased tissue.

In addition to KCs, unique populations of both DCs and NK cells have been identified in single-cell RNA sequencing datasets, in addition to previously identified populations of DCs and liver-resident NK cells (Table 1),⁴⁹ and it is likely that further liver-specific immune cell subpopulations will be identified as this technology matures. Beyond immune cells, it is evident that zonal and disease-specific populations of hepatocytes, endothelial cells, and stellate cells are also present within the liver.^{44,47,50} The technological advances that have enabled the analysis of transcriptional differences across these zonal regions within the liver are now also being applied to resident immune cell populations to uncover further immune cell heterogeneity associated with different regions of the liver.

THE HOMEOSTATIC RESPONSE TO STERILE INJURY IN THE LIVER

Inflammatory activity in the liver that is stimulated in the absence of foreign microbial antigens can occur in response to a wide range of stimuli and is commonly referred to as sterile inflammation.⁵¹ This inflammatory response can lead to the pathological production of reactive oxygen species (ROS), lipid-derived metabolites (retinoic acid and endocannabinoids), and damage-associated molecular patterns (DAMPs), which amplify inflammatory signals through TLRs, nuclear/neuronal receptors, and the inflammasome (Fig. 2). While maintaining an overall tolerogenic environment, the healthy liver is well equipped to restore homeostasis after disturbances. Large populations of innate immune cells clear senescent, injured, or apoptotic cells, as well as microbes and their associated products.²⁴ Activated neutrophils and cytotoxic cells that are no longer responsive to inflammatory signals or that have already completed their immunologic functions and need to be removed from the circulation migrate to the liver to die via apoptosis. This

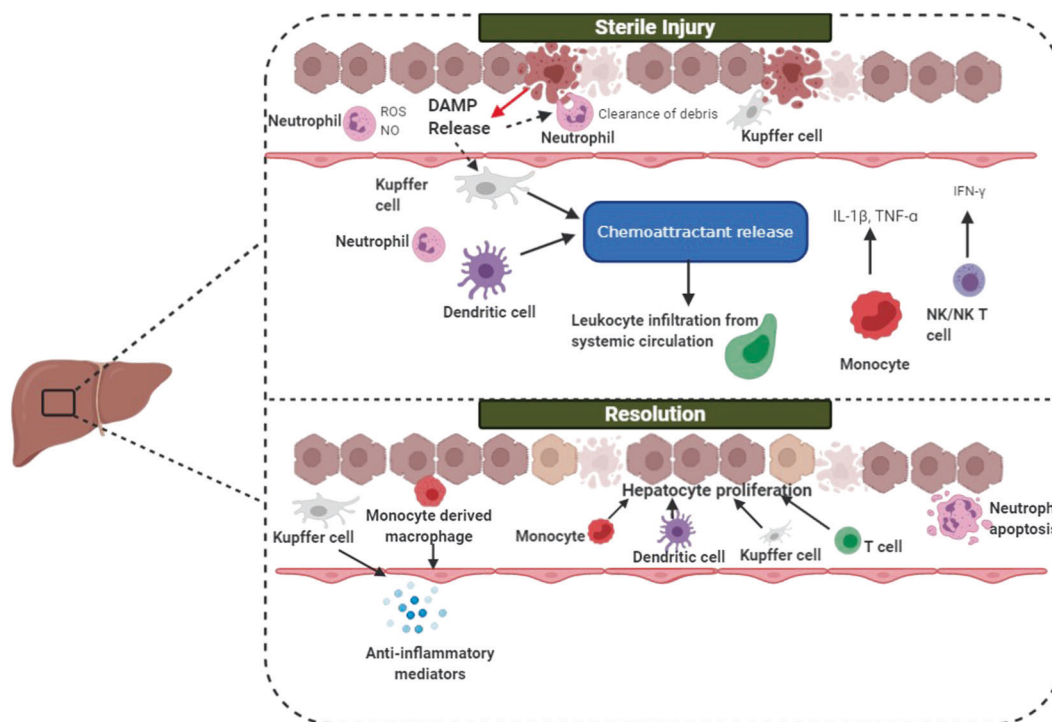


Fig. 2 The acute response to sterile injury in the liver. During liver injury, hepatocyte death causes DAMP release, resulting in Kupffer cell activation, neutrophil recruitment to the site of injury and the activation of hepatic stellate cells, leading to cytokine secretion. Kupffer cells and neutrophils release inflammatory mediators and begin to phagocytose necrotic debris. T cells, dendritic cells, and Kupffer cells facilitate leukocyte infiltration from the systemic circulation by releasing chemoattractants such as CCL2 and IL-17. NK and NKT cells release IFN γ , stimulating the release of proinflammatory mediators. During resolution, monocytes acquire a reparative phenotype, and macrophage reprogramming occurs in a subset of macrophages that exhibit a resolution phenotype. Monocytes migrate across the liver sinusoidal endothelial cell layer, differentiating into macrophages to either replenish the Kupffer cell pool or restore homeostasis by secreting anti-inflammatory cytokines and promoting angiogenesis. Following injury, a restorative macrophage phenotype emerges, promoting neutrophil apoptosis. Hepatocyte proliferation occurs to replace the lost parenchyma¹³¹

detoxification mechanism or resolution is largely mediated by KCs, which are the first cells in the liver to encounter cells and molecules from the systemic circulation. High quantities of microbial DAMPs and MAMPs arriving via portal venous blood bind to PRRs expressed on KCs and some hepatocytes, facilitating microbial phagocytosis and degradation without initiating the overwhelming immune response that is usually stimulated by PRRs.^{52,53} In a cirrhotic mouse model, endotoxin accumulation appeared to result from decreased KC uptake and low levels of TNF alpha release.⁵⁴ In this context, hepatic detoxification ensures that inflammatory antigens from the gut do not travel to the rest of the body, causing excessive immune activity.⁵⁵ While it is unsurprising that failed detoxification mechanisms are pathognomonic of hepatic failure predicated on the assumption of immunoregulatory disturbances, few studies have defined the inflammatory interplay in hepatic detoxification and its clinical implications.

Some NK cells respond rapidly to injury following direct lipid recognition or cytokine secretion by other immune cells, leading to the downstream accumulation of additional macrophages and neutrophils in the liver.⁵⁶ In murine models, type 1 NKT cell-deficient mice do not exhibit neutrophil and macrophage infiltration in response to injury and display hepatic resistance to IRI or a high-fat diet, suggesting that one subset of NKT cells promotes injury.^{57–59} In contrast, sulfatide-reactive type 2 NKT cells play an opposing role in hepatic IRI by protecting against injury, and a novel anti-inflammatory mechanism has been proposed to exploit this process and ultimately result in NKT cell recruitment in response to liver injury.⁵⁹

This response to acute sterile inflammation is physiological and self-limiting and results in the restoration of tissue homeostasis.

However, the ongoing presence of the stimuli driving chronic sterile inflammation leads to pathology. These stimuli include alcohol metabolites that can induce hepatocyte cell death and excessive fat deposition within hepatocytes, resulting in lipotoxicity and oxygen depletion during ischemia and reperfusion that leads to ischemic damage.

NONALCOHOLIC STEATOHEPATITIS (NASH)

Changes to the hepatic immune environment characterize the development of NASH, and murine and animal studies have been used to define the specific immunological events that occur throughout the clinical course of NASH (Fig. 3). Innate immune activation and dysregulated inflammation have both been implicated in the loss of tissue homeostasis and are a subject of considerable focus. Lipotoxicity, which results from aberrant lipid accumulation during prolonged nutrient excess or obesity exceeding adipose tissue capacity, underlies the generation of several harmful signaling intermediates.⁶⁰ When storage is overwhelmed, lipid deposition occurs in ectopic sites, such as the liver, interfering with local immune regulation and resulting in a cycle of persistent metabolic dysregulation or “metainflammation”^{60,61} Inflammatory immune activation within the liver induces the infiltration of several immune cell subsets and drives the progression of hepatic injury.

KCs appear to play a pivotal role in NASH initiation and pathogenesis.⁶² Concurrent activation of KCs is seen following the introduction of a high-fat diet or methionine/choline-deficient diets in several murine models, indicating the early involvement of KCs in NASH.^{63,64} Human studies have also corroborated these findings, and KC activation and accumulation were observed in

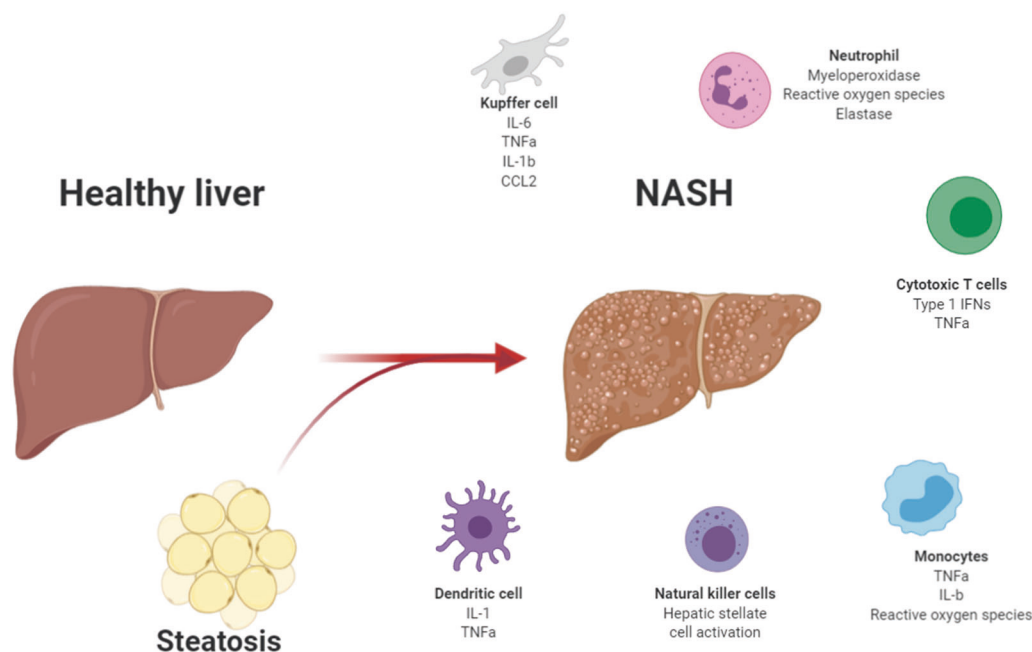


Fig. 3 Pathogenic immune mechanisms in NASH. NASH involves immune cell populations that functionally interact with each other. Increased fatty acid levels and lipotoxicity directly activate inflammation and result in hepatocyte injury and the release of DAMPs, triggering an amplified inflammatory response. Kupffer cell activation stimulates the subsequent release of several proinflammatory cytokines that activate monocytes and neutrophils. These cells exacerbate liver injury by secreting reactive oxygen species and profibrotic signals that promote fibrosis. Cytotoxic T cell activation contributes to perpetuating inflammation by the production of large amounts of IFN γ and TNF α , and natural killer cell activation directly stimulates hepatic stellate cells and hepatocytes, causing further inflammation and inducing profibrotic signals

NASH samples, supporting the direct role of KCs in lipid processing and metainflammation.⁶⁵ Thus, KC activation could be linked to direct lipid uptake or lipid metabolism and the resultant lipotoxicity that presents as foreign particles.⁶⁶ In human studies, KC expansion was a key step in hepatic inflammatory initiation that, through a complex inflammatory network, resulted in the downstream activation of other components of the NASH complex.⁶⁷ Furthermore, there is additional evidence to suggest the pathological role of KCs in hepatic insulin resistance upon activation, further suggesting the intricate interplay of immunological events and metabolic pathways in liver disease.⁶³

The process by which circulating monocytes differentiate into macrophages upon arrival in the liver environment is poorly understood; however, monocyte infiltration may impact some of the inflammatory responses observed in NASH. In response to hepatic injury, Ly-6C^{hi} monocytes differentiate into Ly-6C⁺ macrophages, stimulating proinflammatory and profibrogenic processes.⁶² In murine models, diet-induced hepatic steatosis induced Ly-6C^{hi} monocyte recruitment via CC-chemokine receptor 2 (CCR2), which is largely expressed by KCs and HSCs.⁶⁸ Activated monocytes were recruited following hepatic injury and guided by the expression of CC-chemokine ligand 2 (CCL2), and reduced inflammatory macrophage levels were observed in CCL2-knockout mice with MCD diet-induced NASH.⁶⁹ In contrast, an anti-inflammatory, tissue-protective Ly-6C^{low} phenotype can emerge when apoptotic hepatocytes are phagocytosed, and these cells act to restore tissue homeostasis.⁷⁰

Moreover, KC polarization into M1 and M2 phenotypes further reveals the cyclical and overlapping proinflammatory and immunoregulatory events in NASH, reflecting the tolerogenic and immunogenic nature of hepatic physiology.^{71,72} For example, M1 phenotype activation results in the production of several proinflammatory cytokines and chemokines, such as TNF- α , IL-1 β , IL-12, CCL2, and CCL5, inducing additional hepatocyte injury and

DAMP release, which further exacerbates KC activation and results in a vast influx of monocytes.^{11,62} In contrast, the role of M2 phenotype cells, although largely undefined in NASH, involves inflammatory resolution and healing processes to counteract the initial recruitment of various cellular populations.^{62,72} To date, few studies have examined M2 phenotypic changes in NASH compared to non-NASH in humans, although there is evidence to suggest PPAR- δ involvement in M2 activation, which may offer a therapeutic strategy in the clinical management of the entire NAFLD disease spectrum.⁷³ In addition, mice fed a 30-week NASH diet exhibited substantial KC loss driven by genetic changes in KC enhancers and subsequent cell death.⁷⁴ Consequently, increased expression of TREM2 and CD9 in several KC phenotypes was observed and could indicate the severity of steatosis and inflammatory injury in NASH.^{75,76} Recent single-cell analysis has further performed and identified the presence of scar-associated macrophages in murine NASH models and cirrhotic human livers.^{47,75} This TREM2⁺CD9⁺ macrophage subset induced collagen expression in HSCs, and their frequency correlated with fibrosis scoring and disease severity in NASH.^{47,48}

Collagen deposition resulting from hepatic stellate cell activation has been linked to the effects of profibrogenic factors through KC activation, and in vivo research has indicated that KC depletion could attenuate fibrosis development in the late stage of the disease.⁷⁷ Small animal and patient studies have reported increased levels of activin-A, a multifactorial cytokine belonging to the TGF β superfamily, in liver disease, leading to the downstream activation of KCs.^{78,79} In a mouse model, the expression of proinflammatory molecules after activin-A KC activation further induced HSC-dependent fibrogenesis, which was observed in the later stages of NASH cirrhosis.⁸⁰ KC activation promotes HSC transdifferentiation into myofibroblastic HSCs, which leads to the production of extracellular matrix elements, cytokine secretion, and alpha smooth muscle actin expression, which are necessary for the progression of fibrosis and hepatic scarring.⁹

In addition to in situ macrophage activation and monocyte recruitment, hepatic infiltration by additional cellular subtypes, such as lymphocytes and neutrophils, contributes to the underlying inflammatory disturbances that occur in NASH (Fig. 3).⁶¹ Following highly orchestrated immune processes in NASH, large amounts of proinflammatory cytokines and chemokines mediate the influx of additional populations of CD4⁺/CD8⁺ T and B lymphocytes, which further enhance macrophage activation and inflammatory activity.⁶¹ Increased levels of both CD4⁺ and CD8⁺ populations are found in the blood of NASH patients, and the degree of portal inflammation due to CD8⁺ cells correlates with disease severity.^{81,82} In high fructose murine models, CD8⁺ deficiency was associated with steatosis resistance compared to that of controls.⁸³ Recent evidence has suggested a role of B lymphocytes in regulating T-cell activation in NASH, although some of the evidence has been conflicting.⁶¹ It is unclear whether B-cell lymphopoiesis is enhanced or compromised in obesity states; however, B cell-derived IL-10 has been shown to inhibit proinflammatory cytokines in metabolic syndromes.⁸⁴

ISCHEMIC LIVER DAMAGE

The dual blood supply of the liver consists of oxygenated blood delivered via the hepatic artery, which accounts for 20% of hepatic inflow, with the remaining 80% delivered by the portal venous system. The healthy liver, therefore, has characteristic zones of high and low oxygen levels, forming an oxygen gradient that is necessary for hepatic function, which is described as liver "zonation" and is required to maintain the structural integrity of hepatocytes^{85,86} (Fig. 4). The concentration of oxygen decreases along the sinusoids as blood passes from Zone 1 (periportal zone) through to Zones 2 (transitional zone) and 3 (perivenous zone). This understanding of zonation has important implications for homeostasis, and various metabolic capacities can be preferentially found in specific zones.⁸⁷ Although the underlying regulatory pathways of oxygen zonation within the liver remain to be fully elucidated, complementary metabolic activities and spatially separated opposing pathways associated with carbohydrate, amino acid, and lipid metabolism have been shown to occur within distinct oxygen zones.⁸⁸ In ischemic injury, the absence of oxygen disrupts this finely tuned gradient, resulting in several functional changes at the cellular level in response to oxidative injury that counteract and control the damage (Fig. 5). Hypoxia inducible factors (HIFs) play critical roles in maintaining homeostasis. However, despite the liver being exposed to variable and often low oxygen tensions, hypoxic responses are not induced in

normal conditions.^{55,86} During prolonged periods of severe ischemia, as seen during transplantation and hepatic resections, HIF activation supports oxygen-independent ATP generation to mediate cellular processes and upregulates cell preservation mechanisms through antioxidant and antiapoptotic systems that facilitate cell survival. Unsurprisingly, hypoxia results in a proinflammatory response by increasing the expression of IFN- γ , MHC II, and costimulatory molecules in hypoxic macrophages to induce T cell-driven cytokine production.^{89,90} In addition, both human and murine models have been used to study adaptive immune responses and T-cell recruitment in inflammatory hypoxia, and selective FoxP3 induction and upregulation plays a key role in homeostasis by eliciting potent anti-inflammatory responses to limit overwhelming hepatic injury via Treg signaling.^{91,92} These data suggest the complementary role of HIFs in hepatic immunomodulation and tolerance, but the details of these mechanisms require further clarification.

KCs that are activated during ischemia produce ROS, TNF α , and IL-1 β , resulting in subsequent leukocyte recruitment, hepatocyte death, and endothelial damage.^{62,93} In addition, ROS and cytokine secretion activate CD4⁺ T cells and NKT cells, which produce IFN- γ , potentiating KC activation.⁹⁴ Widespread cytokine activation also upregulates the expression of T cell-associated cell surface adhesion molecules on SECs and ROS production by hepatocytes, triggering a multicellular immune process that contributes to an overwhelming inflammatory response.^{95,96} The end result is a complex communication network of immune cells that propagate IRI. Hepatic immunity during ischemia is mainly associated with immune restoration and regeneration without generating detrimental immune responses in normal contexts. As mentioned previously, liver zonation and the maintenance of an oxygen gradient are required to support the structural integrity of healthy hepatocytes.⁸⁵ Thus, the pathological consequences of ischemia are particularly apparent during ischemia/reperfusion, which is a widely accepted example of sterile inflammation, and the rapid restoration of oxygen to ischemic tissue triggers apoptosis and oncotic necrosis (Fig. 5).^{97,98} Ischemia can be categorized as warm or cold, depending on the temperature at which the ischemia occurs. While warm ischemia is known to cause major disruptions in molecular signaling and cellular pathways, cold ischemia has been considered protective due to reduced ATP demands for regular molecular processes. Thus, cold ischemia is being utilized in liver surgery, especially transplantation, to minimize the injuries encountered during warm ischemia. In both contexts, the physiological repercussions are proportional to the extent of ischemia and the duration of oxygen blockade. Therefore,

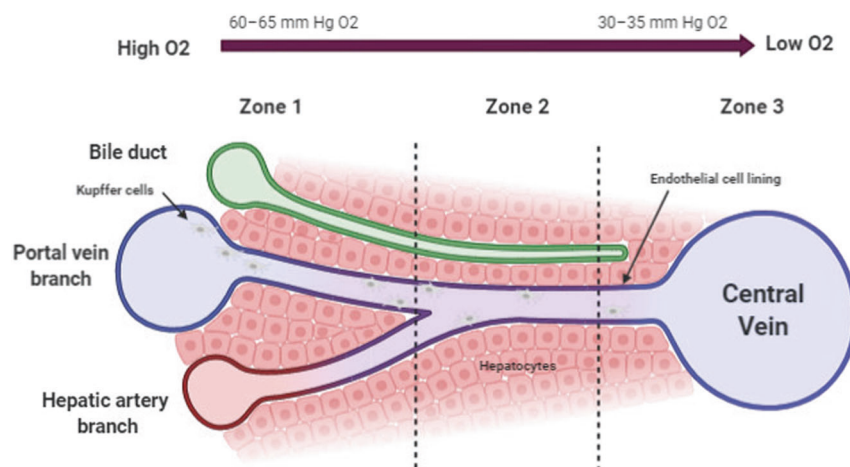


Fig. 4 Oxygenation within the liver. The oxygen gradient decreases in blood as it travels through the hepatic sinusoids (Zone 1) toward the pericentral region (Zone 3), significantly altering the local microenvironment and influencing immune cell localization and activity. Kupffer cells are larger and more numerous in periportal zones than in perivenous zones¹³²

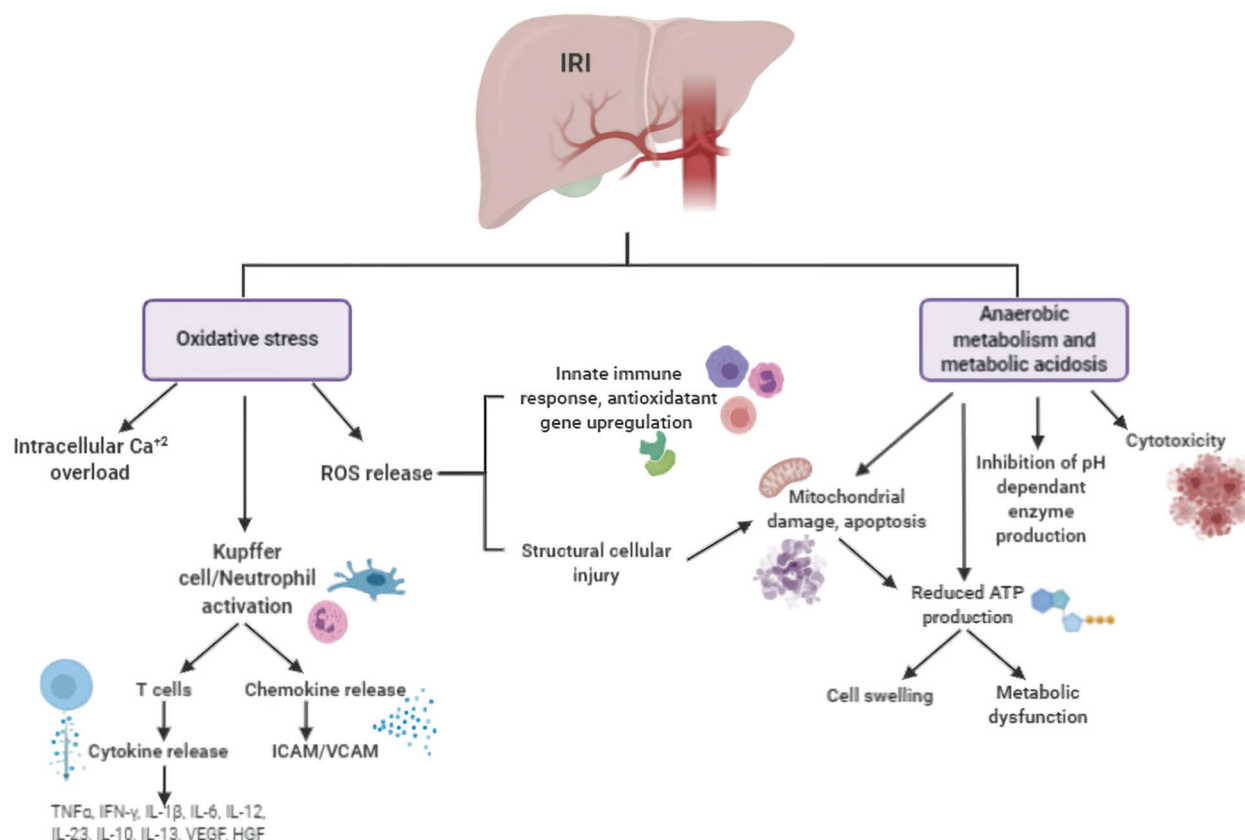


Fig. 5 Pathological immune mechanisms in ischemia-reperfusion injury (IRI). The mechanisms of hepatic IRI are complex and result in a series of cellular responses influencing inflammatory, metabolic, and antioxidant pathways.¹³³ Tissue hypoxia, oxidative stress, and the resulting anaerobic cell metabolism lead to structural cellular injury and a large amount of chemokine and cytokine release to enhance downstream immune cell accumulation. ATP adenosine triphosphate, IL interleukin, ROS reactive oxygen species, IRI ischemia-reperfusion injury, IFN-γ interferon-gamma, ICAM intercellular adhesion molecule, VCAM vascular cell adhesion molecule, TNF tumor necrosis factor, HGF hepatocyte growth factor, VEGF vascular endothelial growth factor

ischemic injury and IRI, although oftentimes used synonymously, are distinct entities. During ischemia, hepatocytes are immediately disrupted, and metabolic processes shift from oxidative phosphorylation to glycolysis.⁹⁸ The subsequent ATP depletion and calcium accumulation lead to necrosis in the absence of ROS, which distinguishes this process from the pathological processes of IRI.^{98,99} In routine hepatic surgery, the ischemic time is limited, and severe ischemic injury can be avoided. The main inflammatory response occurs during reperfusion and tissue reoxygenation, whereby hepatocyte leakage results in DAMP release and subsequent KC activation, which is the main driving factor of ROS production.^{98,100} In addition, activation of the complement system and its components, of which several are potent neutrophil activators, contribute to the widespread inflammatory cascade during reperfusion.^{101,102}

The liver parenchymal condition strongly determines the extent of ischemic injury, and several animal models have been used to establish reduced hepatic regeneration and extensive IRI injury in the background of chronic liver disease. It has been demonstrated in murine models that steatotic and cirrhotic livers exhibit devastating inflammation and hepatic injury following IRI with a reduced abilities to recover normal cellular function and healing.^{93,103,104} Molecular aberrations in older livers are likely to weaken immunotolerogenic responses to ischemic injury. Older livers exhibit reduced tolerance to IRI and a consistent inability to regenerate, partly explaining why younger donors are considered optimal in the context of transplants.^{105,106} In a mouse model, mature adult mice displayed significantly reduced neutrophil accumulation, suppression of the inflammatory transcription

factor NF-κB and almost complete absence of macrophage inflammatory protein-2, which is necessary for neutrophil recruitment.¹⁰⁷ Tissue remodeling roles of neutrophils have also been described in IRI. In a model of sterile liver injury, neutrophils were shown to dismantle injured vessels and create channels for new vascular growth,¹⁰⁸ while in a chronic liver injury model, neutrophils promoted fibrolysis and suppressed the development of fibrotic lesions.¹⁰⁹

A significant proportion of harvested donor livers are rejected because of fat deposition and damage that is likely caused by fat-induced sterile inflammation. Fat-related damage may significantly enhance IRI during transplantation and the subsequent loss of the graft.¹¹⁰ A major aim in liver transplantation research is to provide protection to minimize ischemic reperfusion injury, particularly in marginal livers, allowing them to withstand surgery.¹¹¹

NEW THERAPEUTIC TARGETS AND STRATEGIES FOR IMMUNE-MEDIATED LIVER PATHOLOGY

NASH

Accumulating evidence implicates the hepatic microenvironment and immune repertoire with chronic liver injury, particularly when the balance between inflammation and tolerance is disrupted, resulting in important clinical consequences. The increasing prevalence of metabolic syndrome and consequences such as NASH pose several challenges that demand novel therapies to target the inflammatory microenvironment.⁵ The altered immune environment in NASH has been a topic of considerable focus in

Table 2. Immunotherapeutic strategies that are currently under investigation for the treatment of immunopathological liver disease

Therapeutic strategy	Targeted disease	Potential mechanism	Refs
Oral anti-CD3 mAb	NASH	Induction of CD4 ⁺ latency-associated peptide (LAP)-positive Tregs. Increased levels of TGF- β	92,93
Bovine colostrum (IMM-124E) (NCT03042767)	NAFLD	IgG antibody against bacterial LPS to prevent TLR4 signaling and cytokine release	116
Centriciviroc (NCT02217475)	NASH Liver fibrosis	Blockade of C-C chemokine receptors type 2 and 5	94,95
Hypothermic ex vivo machine perfusion (NCT04203004)	Ischemia-reperfusion injury in liver transplantation	Hypothermic oxygenated perfusion with cytokine filtration of TNF α , IL-6, IL-8, and endothelin-1	98

recent times, and several therapeutic strategies have been proposed to decrease local hepatic responses without compromising overall immunity. Inflammatory genes are overexpressed in NASH, and an array of proinflammatory factors are secreted by KCs, NKT cells, HSCs, DCs, monocytes, and lymphocytes, perpetuating a cycle of injury and DAMP release, which intensifies the innate immune response.¹¹² To dampen T-cell responses during NASH, the induction of oral tolerance by the administration of anti-CD3 mAbs is currently being explored in murine models.⁶¹ In a leptin-deficient model of fatty liver, oral anti-CD3 mAbs alleviate hepatic fat accumulation, improve liver enzymes, and normalize serum glucose levels.¹¹³ Thus, a randomized, controlled clinical trial was conducted to determine the efficacy of oral anti-CD3 mAbs on human NASH subjects over a 30-day period (Table 2).¹¹⁴ Treated patients demonstrated significant reductions in triglyceride levels and liver enzymes and elevations in TGF- β , indicating anti-inflammatory augmentation. Overall, this work highlights the important role T-cell responses play in NASH and suggest a therapeutic strategy for immune modulation. Further research is warranted to determine the utility of anti-CD3 mAbs in all stages of disease and potential interactions with the overlapping pathways seen in metabolic syndrome.

In addition, the role of endotoxin in the inflammatory pathophysiology of NASH has long been hypothesized, and in human studies, increased plasma IgG levels were found in patients with biopsy-associated disease and correlated with disease severity.¹¹⁵ Following this discovery, hyperimmune colostrum preparations against bacterial LPS alleviated several metabolic abnormalities associated with NASH, primarily lessening chronic inflammation and improving insulin resistance.¹¹⁶ This strategy is currently being tested in pediatric patients with NAFLD for which no approved therapeutic intervention exists (NCT03042767) and follows the promising results observed in adult experiments in which IMM-124E, a hyperimmune bovine colostrum enriched with IgG against *Escherichia coli*, resulted in improvements in clinical parameters for alcoholic hepatitis (NCT01968382).

In murine and human models, monocytes have emerged as key mediators of hepatic injury through chemokine regulation and appear to be attractive targets for therapeutic interventions. Pharmacological blockade of CCR2 blocked macrophage infiltration, steatohepatitis, and fibrosis in mice with chronic liver injury.^{117,118} These results await successful translation into clinical use in affected patients, and the Centaur Trial (NCT02217475) is currently studying the effects of antagonizing CCR2 and CCR5 in a phase 2b clinical trial of NASH patients and fibrosis.

Liver surgery and ischemic injury

In hepatic surgery, the proinflammatory cytokines produced following IRI, especially in the context of transplantation, are potential therapeutic targets. The complexity and interactions of inflammatory factors have made it difficult to design a therapeutic strategy that could interact with the overlapping signaling pathways involved. The challenge in liver surgery is to identify agents that could abrogate ischemic insults and IRI within a realistic timeline while also minimizing collateral deleterious effects to the liver and other organs. One of the most significant improvements in transplantation surgery has been in the area of allograft machine perfusion and preservation. In this context, ex vivo machine perfusion strategies to minimize ischemic and inflammatory injury in transplanted livers have gained attention and are slowly being integrated into clinical practice.^{119,120} Two main perfusion approaches exist for the liver: (a) normothermic perfusion, which uses blood or oxygenated perfusates at physiologic temperatures, and (b) hypothermic perfusion using cooled oxygenated fluids.^{121,122} In both approaches, livers are perfused ex vivo immediately following procurement rather than undergoing static cold storage, and at both temperatures, acceptable viability measures and patient survival outcomes have

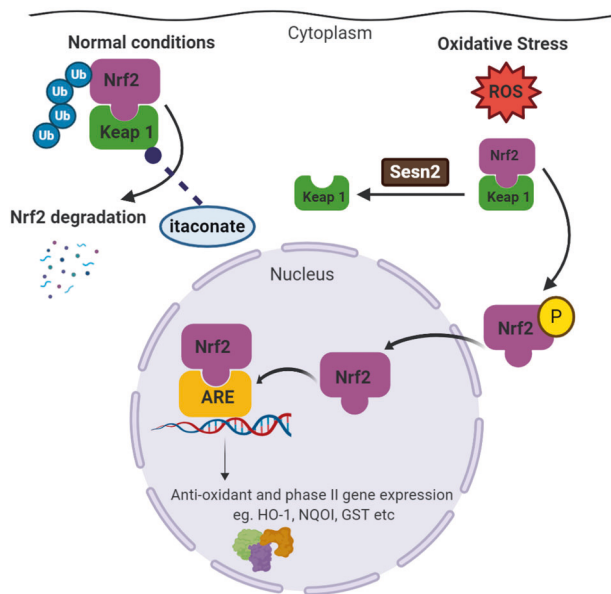


Fig. 6 The Nrf2 activation pathway. Under basal conditions, Nrf2 is found in the cytoplasm bound to the inhibitory protein Keap1, and protein levels are kept low through a ubiquitin-dependent degradation mechanism. During oxidative stress and the release of reactive oxygen species (ROS), structural restriction of the Keap1 protein complex stimulates the release of Nrf2 and its subsequent translocation into the nucleus. Sesn2 (sesn2) is also induced by oxidative stress and facilitates the autophagic degradation of Keap1 to ensure Nrf2 activation. Within the nucleus, Nrf2 functions as a strong transcriptional activator of antioxidant response elements (AREs) in the promoter segments of corresponding genes. Itaconate can alkylate cysteine residues on Keap1, allowing Nrf2 to accumulate in the cytoplasm, translocate to the nucleus, and increase the expression of antioxidant and anti-inflammatory genes¹²⁸

been achieved.^{123,124} Normothermic interventions aim to scavenge ROS, DAMPs, and proinflammatory cytokines using perfusate scavengers.^{121,125} During hypothermic perfusion, metabolic processes are slowed to reduce endothelial cell injury and KC activation.¹²⁶ An adjunct to pre-existing machine technology could be cytokine filtration techniques, which have shown some success in experimental lung perfusion models.¹²⁷ This technique allows for cytokine removal and the clearance of accumulated inflammatory mediators that are associated with worse outcomes in transplantation, and this approach has seen favorable outcomes in allograft function. A similar tool is currently under investigation using novel hypothermic oxygenated perfusion techniques in liver transplantation (NCT04203004) and could potentially expand the donor pool by optimizing high-risk allograft function.

Immunometabolism

More recently, an intriguing link has been made between itaconate and antioxidant expression, suggesting that the former may promote significant anti-inflammatory phenotypes via an Nrf2 mechanism and offering an exciting therapeutic target for sterile inflammation¹²⁸ (Fig. 6). Itaconate has recently been shown to control pulmonary fibrosis in a murine model by decreasing fibroblast activity, and this antifibrotic effect may be a viable therapeutic strategy in hepatic fibrosis in which the inflammatory cascade results in concurrent fibroblast activation and wound healing.¹²⁹ An important emerging observation is that the same stimulus does not necessarily illicit the same damage in every liver. Some livers seem to be better protected from damage than others. The wide spectrum of immune activity is key to the diverse pathologies seen in response to a given stimulus, and of particular importance is the wide range of pathological injuries that can be

induced in different individuals, indicating the importance of genetic influences. In a recent study, one-third of obese individuals undergoing bariatric surgery did not exhibit liver fibrosis, and a high proportion of these individuals also had normal insulin resistance.¹³⁰ This finding was in contrast to the group who were metabolically unhealthy and who also had liver fibrosis. It is likely that metabolic activity in hepatocytes, particularly the production of Nrf2, which mediates HIF-1 α activation, the induction of protective mechanisms against oxidation, and the activation of the mTOR pathway, is key to protecting against sterile inflammation during IRI. In this context, changing the nutritional regimen of donors prior to organ collection may have a profound effect on the response of the liver to IRI after transplantation.

Concluding remarks

Hepatic inflammatory mechanisms maintain liver homeostasis and protect the body from injury and harmful pathogens while maintaining the liver parenchyma and other organs. Immunological tolerance is balanced by a proinflammatory and anti-inflammatory milieu generated by diverse immune cell populations that play both physiological and pathological roles. While there has been considerable progress in the field of hepatic immunology, several molecular mechanisms and the oftentimes contradictory interplay of immune factors require additional clarification. Although there are many branches of current research aiming to define the hepatic microenvironment, the myriad of local and systemic cellular interactions add to the complexity of immune biology and can obscure the responses to conventional and targeted immune therapies. The clinical implications of an altered hepatic immune environment that often results from global immune aberrations, rather than single isolated deviations from physiological cellular homeostasis, have been a clinical obstacle to date, but promising preliminary results from human studies in the experimental setting are currently awaiting translation to routine care. The challenge in this field is in identifying therapeutic strategies that can target the array of myeloid, lymphoid, and nonimmune cells without compromising the healthy parenchyma.

AUTHOR CONTRIBUTIONS

O.A. drafted the manuscript, designed the figures, and contributed to the main conceptual ideas. M. W. R. contributed to the writing, provided critical feedback, and helped shape the manuscript. C.O.F. designed and directed the review, supervised the work, and contributed to the writing of the manuscript.

FUNDING

This research was supported by an SFI Frontiers Grant.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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ORIGINAL ARTICLE

NRF2 assessment in discarded liver allografts: A role in allograft function and salvage

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Antioxidant defence mechanisms, such as the nuclear factor-erythroid 2-related-factor-2 (NRF2) axis, are integral to oxidative stress responses and ischemic injury. Hepatic antioxidant capacity is contingent on parenchymal quality, and there is a need to develop new insights into key molecular mechanisms in marginal liver allografts that might provide therapeutic targets. This study examines the clinical relevance of NRF2 in donor livers and its response to normothermic machine perfusion (NMP). Discarded donor livers ($n = 40$) were stratified into a high NRF2 and low NRF2 group by quantifying NRF2 expression. High NRF2 livers had significantly lower transaminase levels, hepatic vascular inflammation and peri-portal CD3⁺ T cell infiltration. Human liver allografts ($n = 8$) were then exposed to 6-h of NMP and high NRF2 livers had significantly reduced liver enzyme alterations and improved lactate clearance. To investigate these findings further, we used a rat fatty-liver model, treating livers with an NRF2 agonist during NMP. Treated livers had increased NRF2 expression and reduced transaminase derangements following NMP compared to vehicle control. These results support the association of elevated NRF2 expression with improved liver function. Targeting this axis could have a rationale in future studies and NRF2 agonists may represent a supplemental treatment strategy for rescuing marginal donor livers.

KEYWORDS

basic (laboratory) research/science, biomarker, cellular biology, immune regulation, ischemia reperfusion injury (IRI), liver allograft function/dysfunction, liver transplantation/hepatology, organ perfusion and preservation

Abbreviations: ABI, applied biosystems; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; CD3, cluster of differentiation 3; CD47, cluster of differentiation 47; CD68, cluster of differentiation 68; CK19, cytokeratin 19; CVA, cerebrovascular accident; DAPI, 4',6-diamidino-2-phenylindole; DCD, donation after cardiac death; ECD, extended criteria donor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPPAR1, hepatocyte Paraffin 1; HO-1, hemeoxygenase-1; HTK, histidine-tryptophan-ketoglutarate; IF, immunofluorescence; IRI, ischemia reperfusion injury; LT, liver transplantation; mRNA, messenger RNA; NAFLD, non-alcoholic fatty liver disease; NMP, normothermic machine perfusion; NRF2, nuclear factor-erythroid 2-related-factor-2; OPTN, Organ Procurement and Transplantation Network; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; ROS, reactive oxygen species; SD, standard deviation; SEM, standard error of the mean; UNOS, United Network for Organ Sharing; WIT, warm ischemia time; WUSM, Washington University School of Medicine.

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1 | INTRODUCTION

Liver transplantation (LT) is the only definitive treatment for end-stage liver disease.¹ Obesity-related pathologies and associated liver failure are exploding worldwide. Despite growing needs for LT, less than 10% of global demands are met at the current rate and the elements underpinning this deficit are largely related to geographic variation in disease prevalence and access to treatment.¹⁻⁴ Growing attention is now focused on the discrepancy between organ supply and demand, as the numbers of new entrants onto LT waiting lists continue to rise.⁵ Compounding these issues are the alarming projections of reduced organ quality, primarily due to fat deposition, in the next two decades where donor liver utilization is expected to fall from 78% to 48% if current practices remain unchanged.⁶ Unsurprisingly, these forecasting models parallel the increasing prevalence of obesity, viral hepatitis, alcoholic cirrhosis and the full spectrum of non-alcoholic fatty liver disease (NAFLD).^{1,7} In this setting, increasing the utilization of marginal livers and reducing organ discard, currently at 9%, may alleviate some of the pressures on the LT wait-list.⁵

Amid the increasing organ shortages, the transplant community has proposed meticulous organ salvage strategies and the usage of extended criteria donor (ECD) organs previously considered less than optimal or "marginal."^{8,9} One major obstacle is the increased susceptibility of such allografts to ischemia-reperfusion injury (IRI) leading to higher incidence of early graft dysfunction and organ loss. Thus, current research is focused on identifying mechanisms that may resolve this consequence. A main driving factor in hepatocellular injury is the generation of high levels of reactive oxygen species (ROS) which may cause cell injury and disrupt normal physiology when present in above normal concentrations.¹⁰ In some cases, ROS stimulates the release of nuclear factor erythroid-2-related-factor-2 (NRF2), a transcription factor that regulates anti-inflammatory, anti-oxidant and cytoprotective gene expression.¹¹ At baseline, the NRF2 system is maintained in an inhibitory state and becomes active in response to oxidative stress facilitating antioxidant gene upregulation to combat the ischemic insult.

Several experimental animal studies have highlighted the possibility of using NRF2 modulation for IRI attenuation at LT.¹²⁻¹⁴ The clinical relevance of hepatic NRF2 expression in human studies has not been extensively explored, although, lower levels in older grafts were hypothesized to be responsible for worse IRIs seen in these organs.¹⁵ Levels of NRF2 in marginal allografts have not been analysed nor has the potential for ex vivo normothermic machine perfusion (NMP) to affect NRF2 levels in marginal livers been explored. Machine perfusion has recently shown promise in optimizing marginal liver allografts while simultaneously providing objective allograft viability assessments that determine transplant suitability.^{16,17} Demonstration of a role for NRF2 in the optimisation of marginal liver allografts could provide an additional therapeutic target. Such efforts might help guide appropriate organ selection from discarded allografts and identify which livers can be realistically salvaged for clinical use. The aim of this study was to investigate the

NRF2 axis in discarded human liver allografts, its role as a biomarker of donor suitability and its response to NMP in both human and rat liver samples.

2 | MATERIALS AND METHODS

2.1 | Human samples and NMP

Ethical approval was acquired from the Washington University School of Medicine (WUSM) Institutional Review Board. Samples were obtained from livers procured and declined for transplantation by the liver transplant team at Barnes Jewish Hospital (St. Louis, MO) between 2017 and 2020. In all cases, written informed consent was obtained from each donor family. Livers were procured using a standard surgical technique through a midline laparotomy.¹⁸ After cross clamping the supraceliac aorta, the abdomen is packed with ice and organs are flushed with histidine-tryptophan-ketoglutarate (HTK) solution through the infrarenal aorta and inferior mesenteric vein. In total, tissue from 40 discarded allografts were collected and biobanked at -80°C . Patient details were extracted from the United Network for Organ Sharing (UNOS) database including donor demographics, pre-operative biochemical serum analyses and cause of organ discard. Donor cause of death was defined by adopting the same UNOS and Organ Procurement and Transplantation Network nomenclature for categorical analysis. Our human NMP apparatus is a closed recirculating system, as previously described.¹⁸ Eight livers were placed on the NMP circuit maintained at 37°C for 6-h following a brief storage period at 4°C after procurement.

2.2 | Rat liver procurement and NMP

Animal experiments were approved by the WUSM Institutional Animal Care and Use Committee and Department of Comparative Medicine. Eight male Sprague Daley rats (Charles River Laboratories) were obtained at 5 weeks of age, fed a 60% high fat diet over an 8-week period (Research Diets Inc.) and weighed weekly (Figure S1). Animals were housed under controlled environmental conditions with a 12:12 h light/dark cycle and free access to water. Anesthesia was induced with 4% isoflurane (Sigma) and animals were later maintained on 2% with continuous oxygen flow. The liver was recovered through a midline incision using a modified version of the cuff technique.^{19,20} We used a modified NMP system based on the previously reported Harvard Apparatus.²¹ Livers were placed on NMP using our previously described approach with whole-blood perfusate and cannulation of the portal vein.²² An NRF2 agonist (Oltipraz, ab141957; Abcam; 150 mg/kg) was delivered at the initial 1-hour time point when the system temperature was stabilized,²² and perfusion was maintained at 37°C for an additional 3 h. Perfusate samples were collected at baseline (0 h) and 4-h of NMP for blood gas and biochemical testing. Rat liver tissue was collected at both time points for histological analysis.

2.3 | Western blot and quantitative PCR analysis

Western blot analysis was performed as described previously.²³ mRNA expression was quantified using quantitative PCR on an ABI prism 7000 machine (Thermo). Detailed preparations are described in File S1.

2.4 | Hematoxylin and eosin, periodic acid-Schiff, immunofluorescence staining

Liver samples were fixed with 10% formalin, embedded in paraffin and stained with hematoxylin and eosin according to standard protocols. Biopsies were examined by a histopathologist at WUSM who was blinded to sample identity during the analysis. Three separate features were specifically examined: the degree of fatty infiltration categorized by large droplet and small droplet macrosteatosis, portal triad inflammation using the Banff grading system and IRI parameters with the Modified Suzuki Scoring system.^{24,25}

To assess hepatic glycogen content, periodic acid-Schiff staining was performed (#395B; Sigma). Immunofluorescence (IF) staining was conducted to confirm and locate the expression of NRF2 and assess the degree of portal triad inflammation when present (File S1).

2.5 | Statistical analysis

Data normality was assessed, and parametric tests were used when appropriate. For continuous variables, data were compared using unpaired *t*-tests for normally distributed data and Mann-Whitney *U* test otherwise. The association of categorical variables

(differences for dichotomous variables between groups) was assessed using χ^2 test. The distribution of protein expression was observed and NRF2 expression was dichotomized for the purpose of analysis. Similar to previous studies using NRF2 levels, a median split was performed to classify subgroups based on protein expression with immunoblotting.^{26,27} Livers were stratified into a high NRF2 expression group (above the 50th percentile) and a low NRF2 expression group (below the 50th percentile).^{15,28,29} Demographic and clinical data were compared to determine the relationship to NRF2 expression. GraphPad Prism 8 software (San Diego, CA) was used to generate graphs and data are presented as mean $\pm$ SD or SEM. For all analyses, *p* < .05 was considered statistically significant.

3 | RESULTS

3.1 | NRF2 expression in discarded human livers and donor characteristics

Western blotting analysis revealed that NRF2 expression was highly variable in the 40 donor livers (median age 42 years, range 13–61; male *n* = 25; median relative expression = 1.16, range 0.33–4.41). The median donor BMI was 29.3 kg/m² (range 14.3–50.3 kg/m²) and 20 patients met the criteria for moderate (*n* = 9), severe (*n* = 9), or very severe (*n* = 2) obesity. Anoxia was the most common cause of donor death (*n* = 21), followed by cerebrovascular accidents (*n* = 11) and trauma (*n* = 8). Livers were most often discarded for exceeding Donor after Cardiac Death (DCD) times (*n* = 18), the inability to identify a suitable recipient (*n* = 9), hepatic steatosis (*n* = 7), donors exceeding the upper age limit (*n* = 5), and fibrosis (*n* = 1).

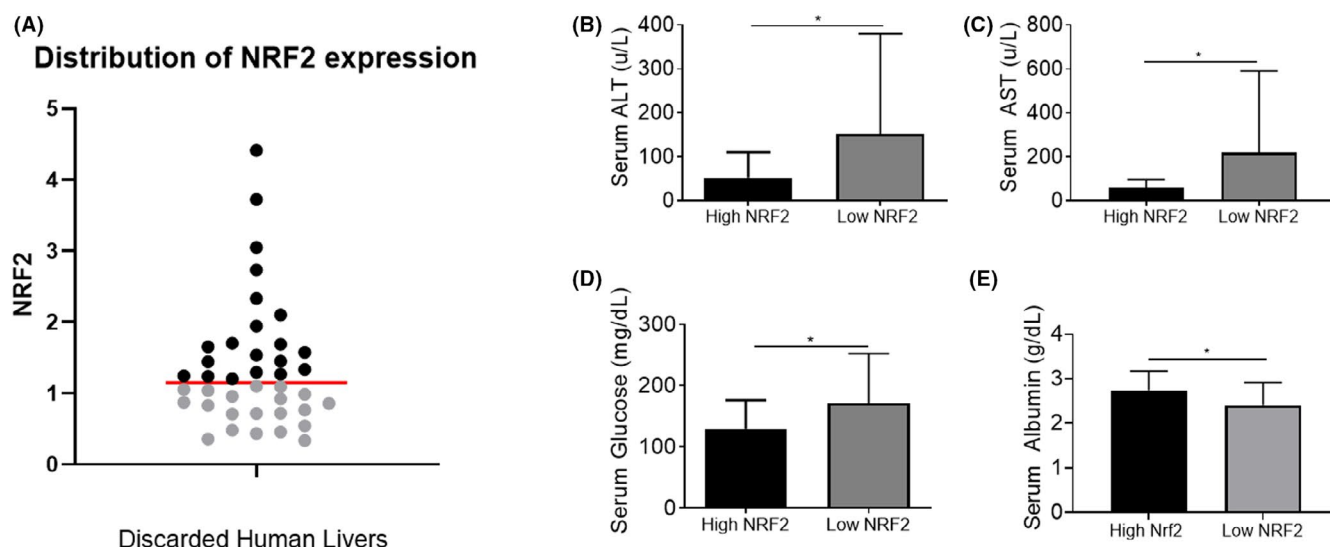


FIGURE 1 (A) Scatter plot of the variable distribution of nuclear factor-erythroid 2-related-factor-2 (NRF2) expression in discarded human livers (*n* = 40). Median value = red line. (B) Biochemical results in livers from high and low NRF2 expression donors. Livers with high NRF2 expression demonstrated significantly lower serum ALT, (C) AST, and (D) glucose but (E) higher serum albumin levels. Data are represented as mean $\pm$ SD. **p* < .05 by *t*-test. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/ajt.16789)]

3.2 | Impact of NRF2 expression on hepatic function

Donor livers were divided into two groups based on high NRF2 (mean expression 1.91 ± 0.88) and low NRF2 (mean expression 0.81 ± 0.27) (Figure 1A; Table 1). No significant differences were observed in warm ischemia times (WIT) between DCD livers with high NRF2 expression (mean 34 ± 18 min) and the low NRF2 group (37 ± 25 min; $p = .31$). Mean donor serum alanine transaminase (ALT) was significantly lower in the high NRF2 group (53 ± 58 u/L vs. 152 ± 228 u/L; $p = .049$) compared to low NRF2 livers. Similarly, aspartate transaminase (AST) enzyme levels were significantly lower pre-operatively (58 ± 46 u/L vs. 200 ± 100 u/L; $p = .045$) in donors categorized in the high NRF2 group (Figure 1B,C). Serum glucose levels were decreased in the high NRF2 expression group (129 ± 47 mg/dl vs. 171 ± 81 mg/dl; $p = .049$) (Figure 1D) while albumin was increased (2.70 ± 0.43 g/dl vs 2.40 ± 0.50 g/dl; $p = .027$) (Figure 2E).

Discarded liver histology revealed a lower percentage of small droplet macrosteatosis in livers with high NRF2 levels but similar amounts of large droplet macrosteatosis (Table 2). Furthermore, livers with high NRF2 levels had lower hepatic artery and portal vein inflammation (Figure 2A; Figure S2) and minimal periportal CD3 T cell positivity was observed ($p < .001$) (Figure 2B) when compared to livers with low levels of NRF2 expression. Lower hepatic glycogen was also observed in livers with high NRF2 levels when compared to livers with low NRF2 levels ($p = .046$) (Figure 2C).

3.3 | NRF2 responses to NMP

Livers ($n = 8$) were selected at random and NRF2 protein was quantified by Western blot. Livers were ranked and divided into two groups based on high and low NRF2 expression levels. NMP responses were again investigated after 6 h (Table 3). Baseline NRF2 expression at 0-h did not differ significantly (Figure 3A). Livers with high NRF2 expression levels demonstrated a 36.5%-fold increase in NRF2 following NMP, while livers with an already low NRF2 expression level saw a further 19.5% decrease in expression levels (Figure 3B,C). On IF, NRF2 was consistently expressed in hepatocytes, cholangiocytes and macrophages (Figure S3–S5).

Following 6 h of NMP, mean ALT was significantly lower in livers with high NRF2 levels (5919.4 ± 4423.6 iU/L) when compared to livers with lower NRF2 levels ($12,001.5 \pm 2961.5$ iU/L; $p = .048$). A similar trend was observed for post-NMP AST (7818.0 ± 4743.0 iU/L vs. $33,405.0 \pm 16,117.1$ iU/L, $p = .029$) and post-NMP alkaline phosphatase (174.0 ± 82.0 iU/L vs. 428.0 ± 25.4 iU/L, $p = .006$) which were both significantly lower in livers with higher NRF2 expression levels (Figure 4A–C). These livers showed acceptable glucose levels at the end of perfusion (265 mg/dL vs. 92 mg/dL, $p = .028$) and hepatic glycogen restoration, which increased, although not significantly. Increased lactate clearance following NMP was

TABLE 1 Demographic details of the population

Variables	High NRF2 expression	Low NRF2 expression
N	20	20
Gender (M:F)	16:4	15:5
Age, median (range)	43 (13–61)	40 (14–61)
Race		
Caucasian	16	17
African American	4	2
Asian	–	1
BMI, mean (SD)	27 (7.8)	32 (7.4)
Lean: steatotic	14:6	8:12
WIT, min (SD)	34 (18)	37 (25)
Donor cause of injury		
Anoxia	11	10
CVA	4	7
Trauma	5	3
Cause for organ discard		
Exceeded DCD time	12	6
No eligible recipient identified	4	5
Steatosis/fibrosis	3	5
Donor age	1	4

also achieved in livers with high levels of NRF2 (0 h = 1.6 mmol/L vs. 2 h = 0.5 mmol/L) when compared to livers with lower NRF2 (0 h = 3.7 mmol/L vs. 2 h = 3.0 mmol/L) (Figure 4D). Livers with high NRF2 levels (Figure 5A) maintained lower hepatic artery inflammatory scores ($p = .02$) after NMP (Table 4) and CD3⁺ cells were significantly reduced when compared to livers with low NRF2 levels ($p = .034$) (Figure 5B).

3.4 | NRF2, steatosis and responses to NMP

Steatotic livers accounted for 45% ($n = 18$) of the total donor group and NRF2 status was analyzed (Figure 6A). Lean livers demonstrated greater NRF2 expression ($p = .002$) when compared to steatotic samples (median steatosis 25% [range 10%–70%]). Steatosis was then graded from 0% to 70% to reflect the studied population sample and NRF2 levels were again assessed. Despite one liver with 70% steatosis, NRF2 expression was markedly decreased beyond 40% of hepatic steatosis (Figure 6B).

Livers exposed to NMP were analyzed based on lean and steatotic status and NRF2 responses were studied. Lean livers expressed higher levels of hepatic NRF2 following 6 h of perfusion when compared to steatotic allografts (mean steatosis $37\% \pm 43\%$), which had decreased or static levels of NRF2 (Figure 6C). Increased nuclear NRF2 intensity was further confirmed in lean livers following NMP (Figure 6D).

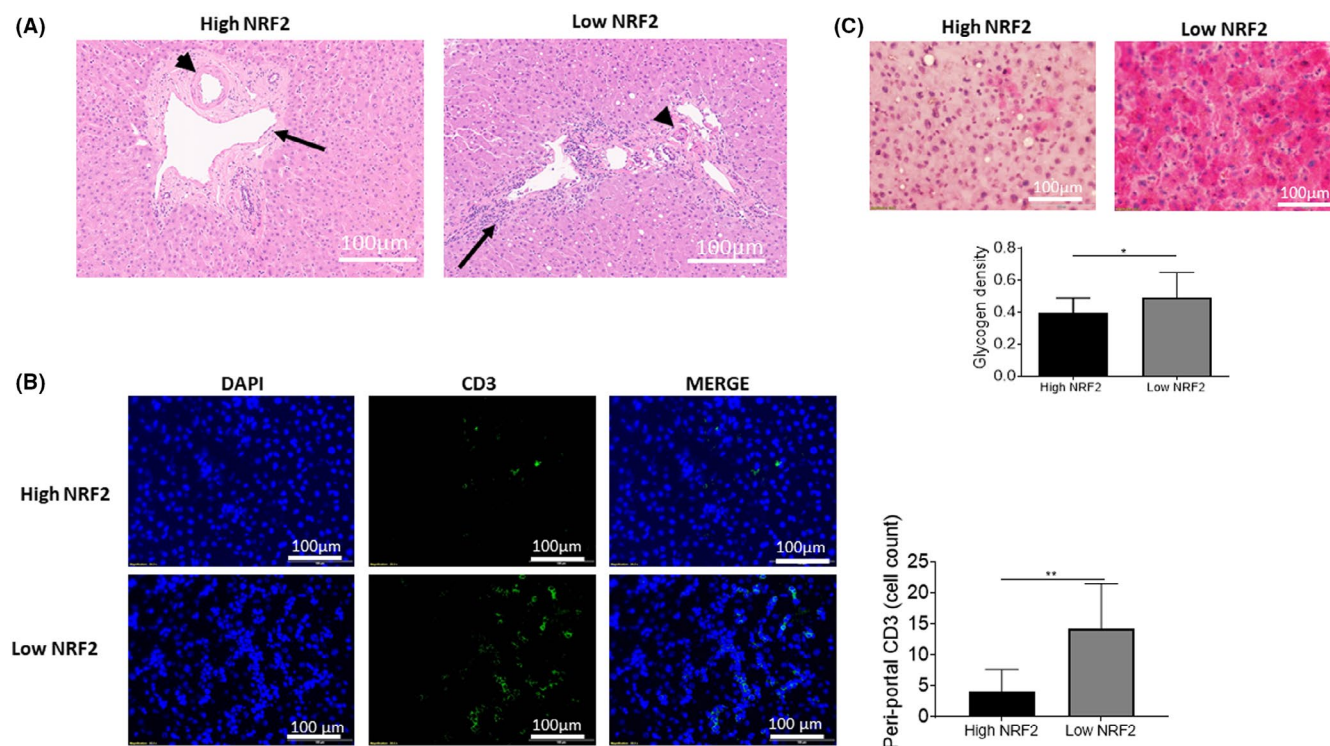


FIGURE 2 Livers in high nuclear factor-erythroid 2-related-factor-2 (NRF2) group have lower inflammation. (A) Representative images of hematoxylin and eosin staining of discarded donor livers. Livers in the high NRF2 group had minimal hepatic artery (arrow heads) and portal vein (arrows) inflammation. (B) Immunofluorescence staining for T cells (CD3, green) and nuclei (DAPI, blue) of high and low NRF2 groups from discarded human livers. Quantitation of immunofluorescence staining revealed increased CD3⁺ cell infiltration around the portal triad in livers from the low NRF2 group. (C) Representative micrographs from periodic acid-Schiff staining (PAS) of discarded human livers from high and low NRF2 groups. Quantitation of relative intensity of glycogen content revealed an increase in glycogen content in livers from the low NRF2 group. Data are represented as mean $\pm$ SD. * p < .05, ** p < .01 by t -test

3.5 | NRF2 augmentation in hepatic steatosis

Having demonstrated that environments with high NRF2 levels were associated with favorable clinical parameters and impaired responses of steatotic livers to NMP, we hypothesized that NRF2 modulation in steatotic livers would result in hepato-protection following ex vivo perfusion. Steatotic rat livers were procured and placed on an NMP system, as in the human model. An NRF2 agonist (Oltipraz) was injected directly into the liver perfusate following the 1-h mark to ensure allograft delivery and reduce systemic loss of drug.

Increased nuclear NRF2 intensity (Figure 7A) was observed in Oltipraz-treated rat livers when compared to the control group (p = .043) (Figure 7B) and hepatic NRF2 (p < .001), *HO-1* (p = .016), and *Gclc* (p = .049) mRNA were increased in Oltipraz-treated livers following 4 h of NMP (Figure 8A–C). Interestingly, NRF2 mRNA levels in Oltipraz-treated fatty livers were comparable to untreated lean livers. Liver enzymes were significantly improved in treated livers that demonstrated a corresponding increase in NRF2 levels after NMP (Figure 8D,E). Potassium was markedly lower following NRF2 agonist treatment (5.6 mmol/L vs 7.3 mmol/L, p < .001) at the end of perfusion. No significant differences were seen in lactate clearance (2.2 mmol/L vs. 1.7 mmol/L, p = .31) and perfusate pH (6.95 vs. 7.01, p = .37) between the compared groups after NMP (Figure S6). Cellular vacuolation (Figure 8F) was significantly increased in

TABLE 2 Histological analysis of discarded high and low expressed NRF2 livers

	Low NRF2 expression	High NRF2 expression	p value
Large droplet macrosteatosis % mean (range)	17.5 (0–70)	8.5 (0–70)	.21
Small droplet macrosteatosis % mean (range)	17 (0–60)	7.6 (0–50)	.04
Necrosis	0.1	0.35	.23
Bile duct inflammation	1.7	1.5	.29
Vascular inflammation			
Hepatic artery	1.5	1.05	.01
Hepatic vein	0.25	0.41	.52
Portal vein	1.45	1	.003
Vacuolization	1.3	1.8	.21
Peri-portal inflammation	1.7	1.5	.32

the treatment group after NMP (p = .049) but no other significant histologic changes occurred despite lower inflammatory scores with NRF2 augmentation (Table 5).

TABLE 3 Donor characteristics of livers undergoing normothermic machine perfusion

Variables	High NRF2 expression	Low NRF2 expression
N	4	4
Donor age, median (range)	51 (49–54)	46 (34–57)
Gender M:F	3:1	3:1
BMI kg/m ² , mean (SD)	25.1 (7.5)	32.4 (2.2)
Donor cause of death		
Anoxia	2	3
CVA	1	1
Trauma	1	–
Cause for organ discard		
Exceeded DCD time	2	2
Donor age	1	1
Steatosis	1	1
Mean WIT, min	56 (34)	26 (4.5)
Lean:Steatosis	3:1	1:3

Abbreviations: BMI, body mass index; CVA, cerebrovascular accident; DCD, donation after cardiac death; WIT, warm ischemia time.

4 | DISCUSSION

Defining the molecular mechanisms responsible for marginal allograft function may help identify therapeutic strategies that will salvage a proportion of discarded organs. The current study presents novel findings regarding marginal liver responses to NMP and positions the NRF2 axis as key to optimal hepatic function (Figure 9). Elevated NRF2 levels were associated with less hepatic injury in both human and rodent samples following NMP, supporting the hypothesis that NRF2 axis amplification may influence or predict discarded allograft function.

In this study, the expression of hepatic NRF2 in discarded human livers was variable. This may be the result of depleted reserves or physiologic decline in some discarded allografts, exacerbating parenchymal injury.^{30,31} To further interrogate this observation, two groups were created and livers with high NRF2 expression demonstrated significantly lower pre-operative transaminases. Higher levels of hepatic NRF2 were also observed in livers with less vascular inflammation. Recent evidence has found that NRF2 deficiency may accelerate vascular senescence and calcification, calling for further investigation into NRF2 targeting in LT.³² Given the ability of

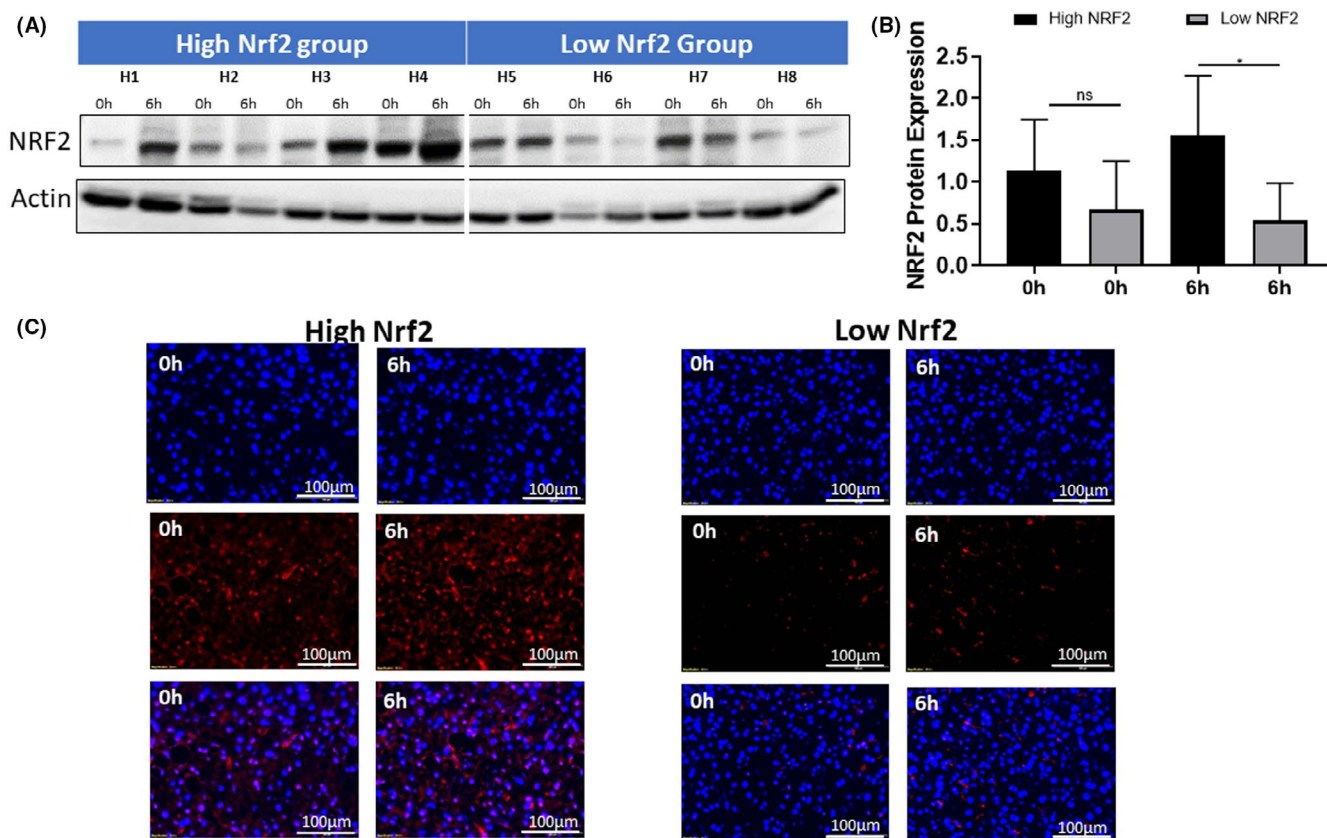


FIGURE 3 Changes to nuclear factor-erythroid 2-related-factor-2 (NRF2) expression following ex vivo normothermic machine perfusion (NMP). Expression of NRF2 was determined by Western blotting at the 0 and 6-h time-point in high and low NRF2 group discarded human livers. (A) Higher NRF2 expression is maintained in high NRF2 group livers after NMP. (B) Densitometric analysis of Western blotting for NRF2 expression demonstrated significantly higher levels of NRF2 expression after NMP. (C) Representative micrographs of immunofluorescence staining for NRF2 (red) and nuclei (DAPI; blue) in high and low NRF2 group discarded human livers. Increased NRF2 expression in livers with higher NRF2 levels which was evident in both nuclear and cytoplasmic content on immunofluorescent staining. Data are represented as mean $\pm$ SD. * $p < .05$; NS, not statistically significant by t -test

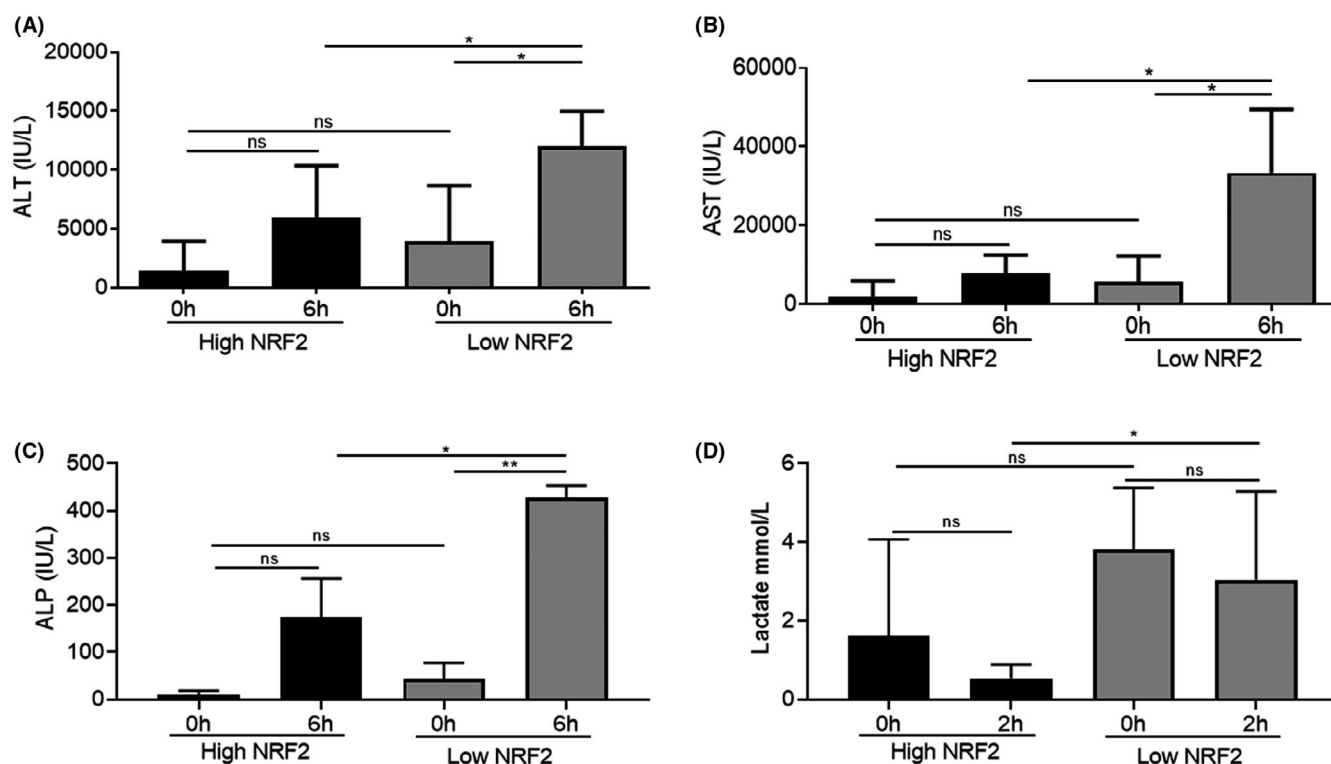


FIGURE 4 High nuclear factor-erythroid 2-related-factor-2 (NRF2) livers have improved liver function after normothermic machine perfusion (NMP). Biochemical assessment of perfusate from livers before and after NMP. High NRF2 group livers had lower (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) alkaline phosphatase following 6 h of NMP (D) and improved lactate clearance at 2 h post-NMP. Data are represented as mean ± SD. * $p < .05$; NS, not statistically significant

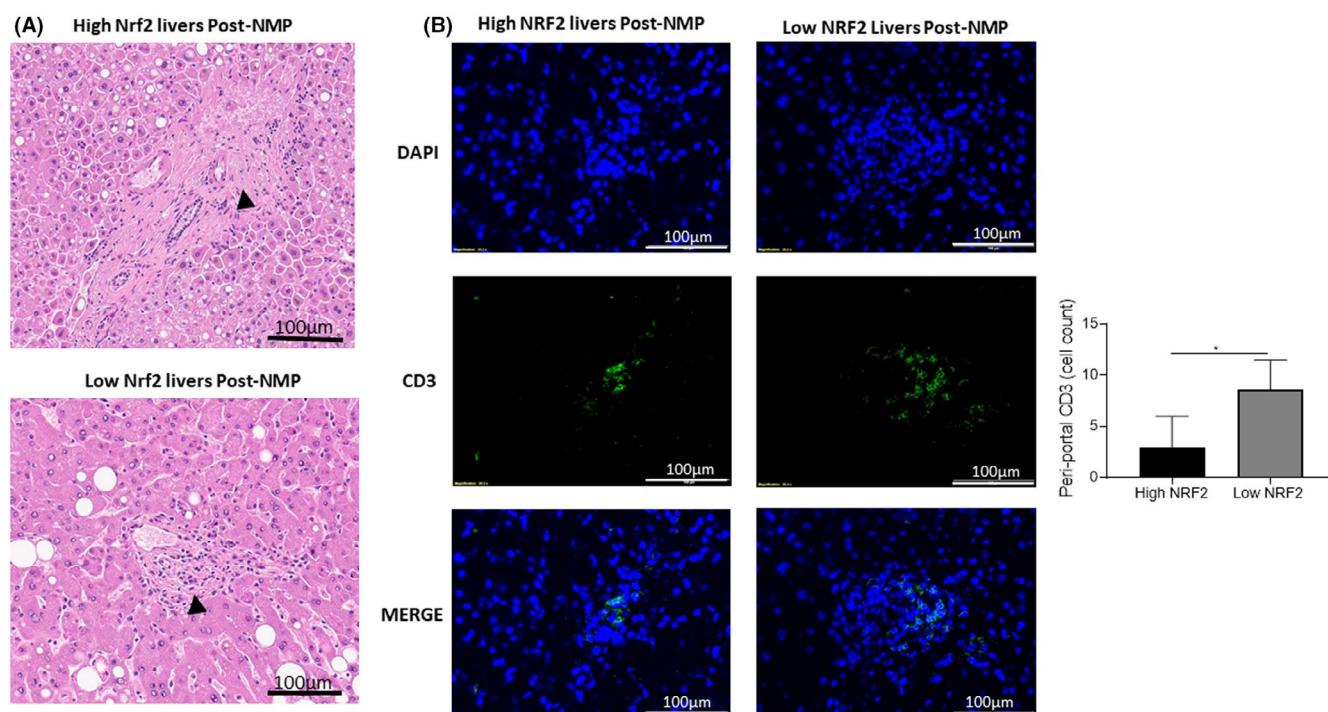


FIGURE 5 High nuclear factor-erythroid 2-related-factor-2 (NRF2) livers have reduced vascular inflammation after normothermic machine perfusion (NMP). (A) Representative images of hematoxylin and eosin staining of discarded human livers after NMP. High NRF2 livers showed lower hepatic artery inflammation (arrow heads). (B) Immunofluorescent staining for T cells (CD3, green) and nuclei (DAPI, blue) in discarded donor livers after normothermic machine perfusion (NMP). Quantitation revealed reduced portal T cell infiltration in high NRF2 livers after NMP. Data are represented as mean ± SD. * $p < .05$ by t -test

NRF2 to moderate both inflammatory and antioxidant pathways, it is likely that this finding, directly relating to hepatic inflow channels, occurred because of optimized tissue repair systems activated by higher NRF2 levels. Increased CD3⁺ cell infiltration was noted in livers with lower NRF2 expression indicating poor resolution of local inflammation. In addition, histological analysis showed that steatosis was significantly higher in low NRF2 livers and this finding parallels the widely reported interplay of the NRF2 axis in hepatosteatosis and the spectrum of NAFLD.^{33–35} While the contribution of small

droplet macrosteatosis to overall allograft function remains debatable,^{36–38} its presence can influence mitochondrial beta-oxidation and overall hepatotoxicity.³⁹ Hence, in the clinical setting, hepatic NRF2 expression appears to associate with more favourable inflammatory, metabolic and functional parameters and could therefore be viewed as an important biomarker to help identify allografts salvageable for transplantation.

To the best of our knowledge, this study is the first to explore the link between hepatic NRF2 expression levels in discarded human livers and their functional response to NMP. High NRF2 livers consistently demonstrated lower liver function test derangements following machine perfusion. Our observation that livers with higher NRF2 expression prior to and following machine perfusion were capable of clearing lactate more effectively may also be a critical factor for allograft selection. Several reports have set transplant eligibility when a lactate threshold of ≤ 2.5 mmol is achieved, correlating with allograft viability and post-operative function.^{16,17,40} There is logic to speculate that the role of NRF2 in antioxidant response and aerobic metabolism could influence blood lactate clearance; however, to date, no study has reported on this relationship directly which would be of immense value in the current liver machine preservation era.^{41–43} High NRF2 livers maintained their immunoprotection of the vascular endothelium, most notably against significant

TABLE 4 Histological variables related to allograft NRF2 expression following 6 h of normothermic machine perfusion

	High NRF2 expression	Low NRF2 expression	p value
Necrosis	0	0.3	.37
Bile duct inflammation	1.3	2	.12
Vascular inflammation			
Hepatic artery	0	1.3	.02
Hepatic vein	0	1	–
Portal vein	0	1	.16
Vacuolization	1	0	.37
Peri-portal inflammation	1	1.3	.37

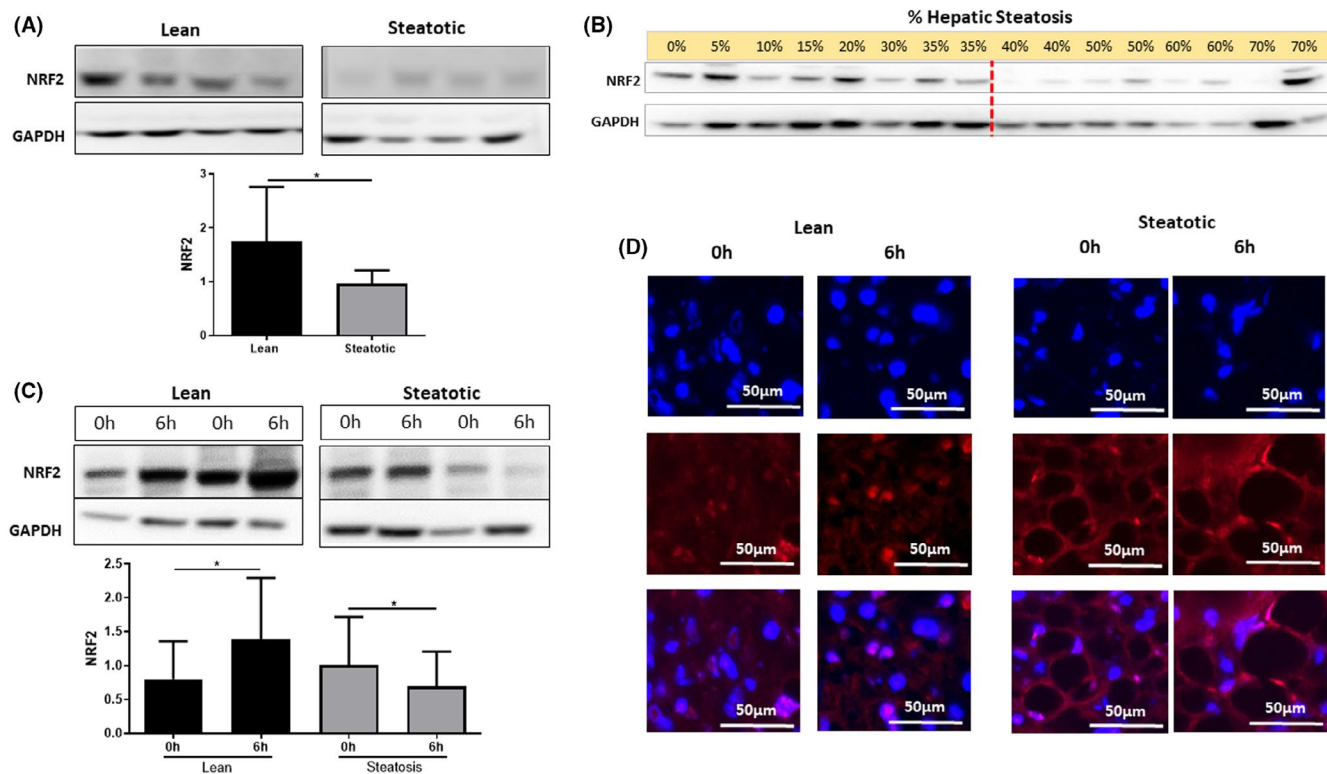


FIGURE 6 Nuclear factor-erythroid 2-related-factor-2 (NRF2) status and hepatic steatosis. (A) NRF2 protein expression levels were greatly reduced in steatotic livers when compared to lean samples on densitometric analysis. (B) Steatotic livers demonstrated variability in hepatic NRF2 expression levels which appeared to drop beyond 40% but some expression was observed in 70% steatosis. (C) Lean livers exposed to 6 h of normothermic machine perfusion demonstrated an increase in NRF2 expression levels on densitometry while steatotic livers lost NRF2 expression. Data are represented as mean $\pm$ SD. * $p < .05$ by t -test. (D) Nuclear intensity of NRF2 increased in lean livers after machine perfusion which was no apparent increase in steatotic samples

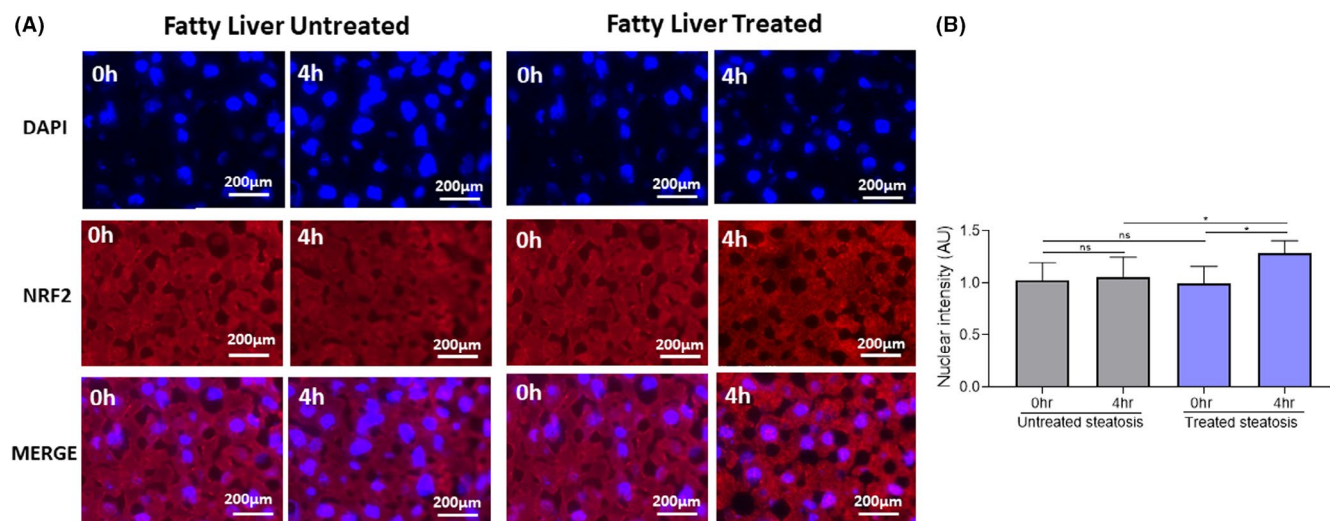


FIGURE 7 Treatment with an nuclear factor-erythroid 2-related-factor-2 (NRF2) agonist increases nuclear NRF2 expression in steatotic livers after NMP. Immunofluorescent staining for NRF2 (red) and nuclei (DAPI, blue) of steatotic rat livers before and after normothermic machine perfusion. (A) Quantitation of immunofluorescence staining revealed increased nuclear intensity in steatotic livers treated with an NRF2 agonist compared with control. (B) Untreated steatotic livers had comparable nuclear levels of NRF2 at 0 and 4 h of machine perfusion, while livers treated with NRF2 agonist had increased nuclear NRF2 expression after 4 h NMP. Data are represented as mean $\pm$ SD. AU, arbitrary unit. * $p < .05$; NS, not statistically significant

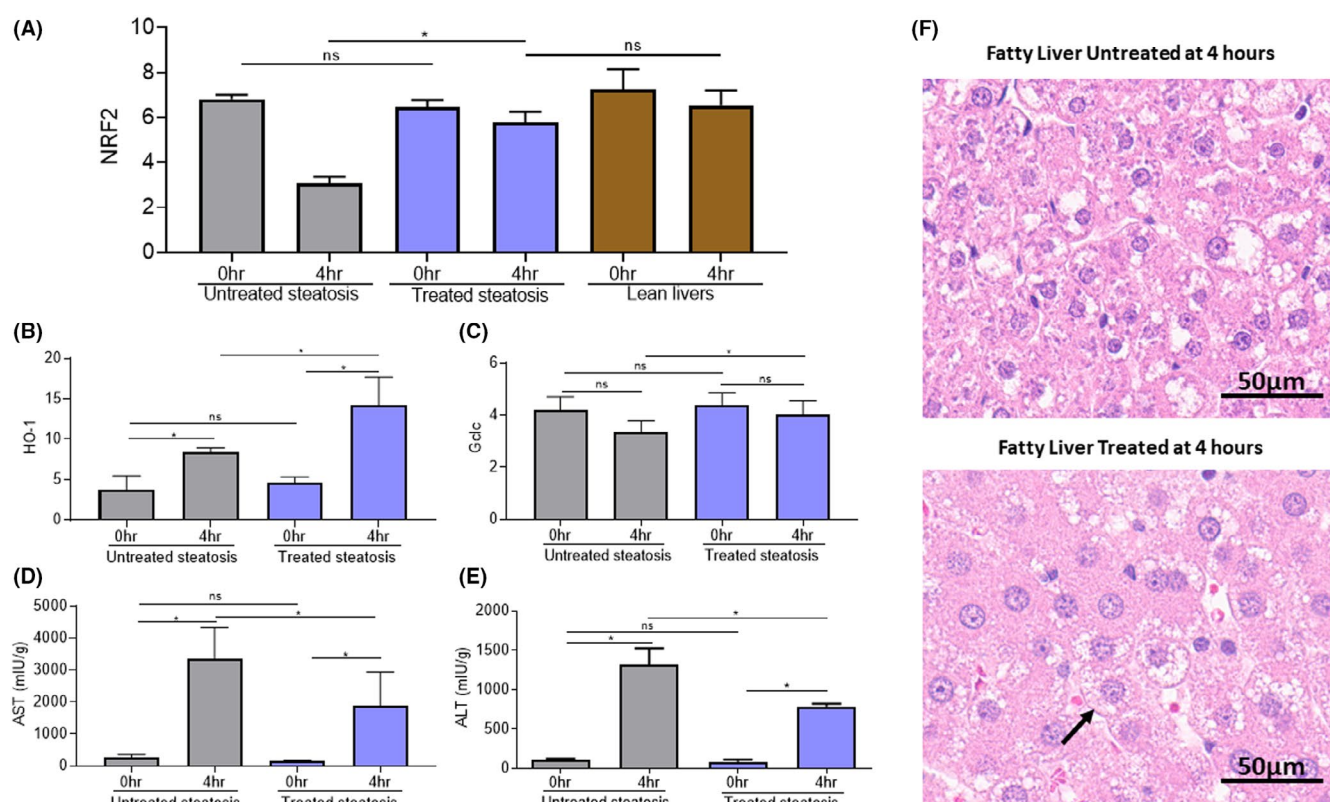


FIGURE 8 Nuclear factor-erythroid 2-related-factor-2 (NRF2) agonist maintains NRF2 expression after normothermic machine perfusion (NMP) and improves liver function. mRNA expression in fatty rat livers treated with vehicle (control) or NRF2 agonist compared to lean rat livers before and after NMP. (A) Relative hepatic NRF2 mRNA (to glyceraldehyde 3-phosphate dehydrogenase) was higher in treated steatotic livers at 4 h and was comparable to Nrf2 levels in lean livers. Treated livers also demonstrated higher (B) hemeoxygenase (HO-1) mRNA and (C) glutamate-cysteine ligase, catalytic subunit (Gclc) mRNA expression at 4 h when compared to controls. Higher Nrf2 levels corresponded with lower derangements in (D) aspartate aminotransferase (AST) and (E) alanine aminotransferase (ALT). Data are represented as mean $\pm$ SEM. * $p < .05$; NS, not statistically significant. (F) Representative images of hematoxylin and eosin staining of steatotic rat livers treated with NRF2 agonist or vehicle control following 4 h of normothermic machine perfusion. Increased vacuolation (arrow) was observed in NRF2 agonist-treated livers after NMP

TABLE 5 Histological variables related to pharmacologic NRF2 augmentation in steatotic rat livers

	Control		Treatment		p value
	30%		26%		
Steatosis, mean (%)	Pre-perfusion	Post-perfusion	Pre-perfusion	Post-perfusion	.75
Necrosis	0	0.5	0	0.5	.50
Bile duct inflammation	1.5	2	1.5	1.5	.35
Vascular inflammation					
Hepatic artery	1.5	1.3	1.5	1	.27
Hepatic vein	0.5	1.3	1.3	0.5	.10
Portal vein	1.25	1	1.5	1	.26
Vacuolation	1	1	2.5	2.5	.049
Peri-portal inflammation	1.5	1.5	1.5	1.25	.26

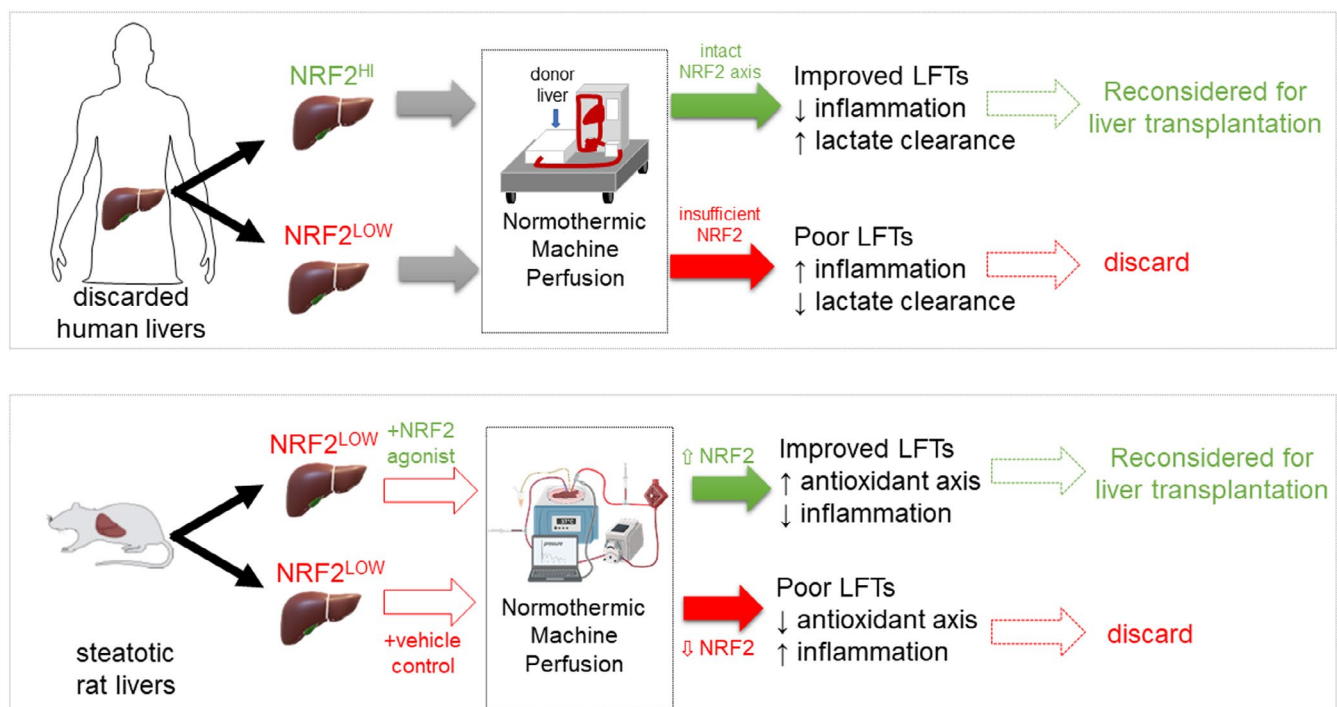


FIGURE 9 Proposed feedback mechanism. Upper panel: Human livers with varying etiologies are discarded from the liver transplant donor pool. When livers are stratified based on hepatic nuclear factor-erythroid 2-related-factor-2 (NRF2) protein expression, livers with high levels of NRF2 (NRF2^{HI}) expression had improved response to normothermic machine perfusion compared to livers with lower levels (NRF2^{LOW}). Less hepatic injury was observed when in NRF2^{HI} livers, and this group represents additional livers which may potentially be salvaged from the discarded organ pool. Future research will determine the suitability of these for transplantation (unfilled green arrow). NRF2^{LOW} livers underwent further injury and remained unsuitable for clinical use (unfilled red arrow). Lower panel: Hepatic steatosis is associated with reduced NRF2 levels. When treated with an NRF2 agonist, steatotic rat livers demonstrated evidence of NRF2 recovery and increased levels of downstream antioxidant genes (hemeoxygenase, glutamate-cysteine ligase, catalytic subunit) following ex vivo perfusion (solid green line). NRF2 agonist also led to improved transaminases in steatotic rat livers. Future research should analyze the suitability of NRF2 agonist-treated livers for transplantation (unfilled green arrow). Untreated steatotic livers demonstrated a further reduction in NRF2 levels following machine perfusion (solid red arrow) and resulted in higher transaminase levels when compared to the treated group. Such livers would appear unsuitable for transplantation due to this degree of hepatic injury (unfilled red arrow)

hepatic artery inflammation. In this regard, NRF2 expression in liver allografts may not only denote improved liver quality, but its upregulation following NMP can be viewed optimistically by protecting

both parenchymal and vascular structures to increase IRI resistance. Interestingly, one of the high NRF2 livers was steatotic, indicating that when livers are grouped into high and low NRF2 expression,

steatosis does not automatically imply marginality. Some rejected steatotic livers may be salvageable for clinical use if a high NRF2 environment is present, and could remain functional from an antioxidant standpoint as implied by our results. These findings can add to the current discussions of ECD definitions and efforts to optimize marginal allografts.^{44,45} The remaining three high NRF2 livers were rejected for transplant for exceeding DCD times, a contentious issue in the field of transplantation. Most transplant centers aim to limit WIT times to less than 30-min^{9,46} and extending beyond this cut-off has been associated with significant graft dysfunction.⁴⁷ Interestingly, WIT did not have any obvious influence on NRF2 status in this study, although this observation may have been limited by a small sample size. Our group has previously shown the benefits of NMP in livers with longer WIT and their ability to demonstrate metabolic and functional parameters comparable to livers subjected to cold preservation.⁴⁸ Hence, the challenge is to accurately identify which discarded livers will derive the most meaningful benefit from NMP and, conversely, those that are likely to be further harmed. Our results suggest that even discarded livers with higher NRF2 expression appear to be much more salvageable prior to and following NMP and, in some instances, meet the viability criteria reported in the literature.

Upregulating NRF2 expression in the rodent model further supports the hypothesis that an NRF2-rich hepatic environment may offer more favourable outcomes. Pre-treated steatotic livers had significantly higher NRF2 elevations following machine perfusion which appeared to correspond with lower transaminases. Additionally, NRF2 upregulation resulted in lower vascular inflammation, although this did not achieve statistical significance and increased cellular vacuolation when compared to control livers. A possible explanation for the latter observation could be that these vacuolated hepatocytes are cells adaptively altering their phenotype to resist injury rather than undergoing degeneration, a finding that has been previously investigated in hepatocellular injury.⁴⁹ This outcome did not result in increased necrosis, and interestingly, other markers of injury were lower in NRF2-treated livers. It is unclear whether NRF2 optimization would have caused histological injury if livers were left to perfuse beyond 4 h and publications regarding vacuolation and cell death are lacking.⁵⁰

Machine perfusion offers a unique opportunity to not only preserve allografts, but also consider the addition of compounds that might enhance allograft viability and quality and exert additional hepato-protective effects. Our group has previously applied NMP in recovering ECD livers.^{18,51} More recently, we have shown that pharmacologic CD47 blockade during NMP can lessen IRI.⁵² Any effort to modulate or increase transcription factor expressions should be met with caution since NRF2 may play a critical role in several cellular functions. Research has shown that somatic mutations in NRF2 may promote cancer cell growth in endometrial, esophageal, lung and bone marrow.^{53–56} Dysregulated activity and constant activation may lead to some undesired effects, especially when the axis appears involved in several pathways.

Notwithstanding these concerns, augmentation of the axis has mediated IRI protection in a number of non-hepatic clinical settings, including cerebral, retinal, intestinal and cardiac tissue.^{57–60} Coordinated NRF2 upregulation has also shown some benefits in treating diabetic nephropathy and naturally occurring NRF2 inducers have been reported to halt acute and chronic inflammatory liver states.^{61,62} While ex vivo NMP is still in its infancy, it may offer a therapeutic window to target the pathway. High NRF2 livers responded positively to 6-h of NMP when compared to low NRF2 livers which saw a further decrease in expression levels. One potential explanation for this could be that an acceptable reserve is necessary to trigger a compensatory response and achieve the favourable clinical environment observed.

We acknowledge some limitations in this analysis. The discarded livers studied have varied ischemia times which likely disrupt a multitude of cellular functions, but reflects the reality of clinical practice and variety of procured liver allografts. Another limitation is the small number of livers placed on the NMP system. The number of ECD livers allocated for research purposes, that are technically and anatomically suitable for ex vivo machine perfusion, is low and using additional livers should be considered to further validate the molecular evidence of NRF2 activation. Future work should also focus on identifying a reliable NRF2 cut-off that correlates with hepatic viability, a task made difficult here for lack of “normal” allografts for analysis. Although, the experimental design did not aim to model transplantation precisely, one remaining hurdle would be to transplant treated livers in the animal setting and investigate whether NRF2 protection is maintained in vivo. Despite these limitations and challenges, the study identifies and confirms the feasibility of targeting NRF2 in discarded marginal livers in a controlled setting and its clinical relevance in allograft function.

5 | CONCLUSION

This study highlights several elements that could aid efforts in minimizing the current rate of organ discard. First, discarded human liver allografts with higher expression of NRF2 have significantly better clinical parameters than NRF2 deficient livers and a small proportion may be suitable for clinical use. Second, ex vivo preservation with NMP could enhance an already intact NRF2 axis and less injury was observed in livers with a positive NRF2 response. In this context, even steatosis did not automatically denote marginality and some fatty livers demonstrated acceptable parameters following NMP that, in the current research climate, may have been considered for transplantation. Third, even on a small-scale animal study, NRF2 modulation has the potential to significantly improve enzyme derangements that usually accompany reperfusion. Further examination into the application of this pathway in marginal allografts that are more susceptible to IRI is warranted. We welcome work toward this objective and in determining the utility of NRF2 as a predictive biomarker or a realistic target for organ salvage and donor pool expansion.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Ahmed O, Xu M, Zhou F, et al. NRF2 assessment in discarded liver allografts: A role in allograft function and salvage. *Am J Transplant*. 2022;22:58-70. <https://doi.org/10.1111/ajt.16789>



Liver transplantation: expanding the donor and recipient pool

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Contributions: (I) Conception and design: All authors; (II) Administrative support: All authors; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Liver transplantation is an exemplar model of complex surgery and the only curative option for patients with end-stage liver disease. Although historically associated with poor outcomes, liver cancer management has also been revolutionised with liver transplantation and in some instances, survival outcomes are comparable to surgical resection. As such, the key elements underpinning the major advances in surgical technique, immunological therapies and allocation policies combined with improved patient and graft survival outcomes have created a huge demand for organ donation. Despite improvements in donor and recipient selection, there is a persistent disparity between organ supply and demand. Candidate wait-list mortality and dropout rates remain problematic and this concern has resulted in increased efforts to expand the donor pool to meet the unmet needs of the population. This is even more challenging when coupled with an ever-growing recipient pool, candidate waiting lists and an ageing population. Over the past two decades, there has been a considerable focus on extended criteria organs, donations after cardiac death and alternative avenues for marginal liver use. With careful donor selection and recipient matching, these livers may help bridge the gap between supply and demand and placate the ever-expanding recipient pool. Here, we present a summary of recent developments by the transplant community addressing the issues of a growing donor and recipient pool.

Keywords: Liver transplantation (LT); expanded criteria; donor pool; recipient pool

Submitted Aug 27, 2020. Accepted for publication Nov 20, 2020.

doi: 10.21037/cco-20-212

View this article at: <http://dx.doi.org/10.21037/cco-20-212>

Introduction

The number of candidates added to the transplant wait list continues to grow. In 2019, 8,896 liver transplants were performed in the United States (US) and the rate has been gradually increasing over the last decade (1). The current annual transplant rate of adult candidates actively listed on the wait list is 58.7% in the US and has increased incrementally compared to previous years. Current 1-, 3- and 5-year survival rates are 91.8%, 83.8% and 76.1%, respectively, and liver transplantation (LT) is

being considered for a growing list of conditions evident by the expanding recipient pool and waiting lists. Despite the benefits of LT, the scarcity of organs is a universal concern and there have been several attempts by the transplant community to propose strategies to overcome the deficit. This has led to the consideration of livers which were previously considered unsuitable for transplantation and efforts to optimize “marginal” organs using innovative machine technology. We, herein, provide a brief overview of the existing approaches by the transplant community to expand the donor and recipient pool for LT.

Extended criteria donation to increase the donor pool

The disparity between supply and demand has led to the consideration of extended criteria donor (ECD) liver allografts or “marginal donors” which do not meet the traditional criteria for organ donation. The aging population, longer life expectancy rates and the rising incidence of several metabolic conditions such as obesity, diabetes and fatty liver disease are all important factors contributing to poor donor quality. Such issues are projected to decrease donor liver utilisation rates from 78% to 44% if ECDs are not included in the pool (2). While these organs were previously avoided due to the fear of primary non-function (PNF) or delayed graft function (DGF), they are now increasingly used with the goal of improving access to transplantable organs (3). The underlying concern is the graft’s susceptibility to ischemia/reperfusion during the transplantation process, adding to graft dysfunction and poor regeneration (4). Despite this risk, ECDs have played an important role in expanding the donor liver pool with compelling evidence demonstrating their ability to reduce wait list mortality and exhibit recipient outcomes comparable to standard liver donations (5,6). There is no universally accepted definition for what constitutes an ECD, however, frequently cited characteristics are advanced donor age, donation after cardiac death (DCD), hepatic steatosis, split liver transplantation (SLT), and donors with an infectious risk or previous malignancy (3,7,8).

Accepting donors with an advanced age

The use of livers from older donors is becoming more frequent in modern practice, despite the concern of DGF during the immediate post-operative period (7,9,10). No clear age cut-off exists and several transplant units are expanding that which is considered an acceptable donor age in an effort to match the increased demand to the ageing population demographic and available donor pool (11). In 2014, 8% of liver donors from the US were 65 years and older and this group increased further to 10% in recent Organ Procurement and Transplantation Network (OPTN) data (12,13). In a report from the European Liver Transplant Registry, Adam *et al.* observed that 29% of donors were older than 60 years and 11% were 65 years or older (14). Some studies have reported acceptable outcomes in livers from older donors. In an early study by Zapletal *et al.* comparing liver allografts from donors both older and

younger than 80 years, comparable results were achieved in the postoperative course and older livers grafts were functionally stable at discharge (15). Several other units mirrored similar results in liver allografts from donors over 60 years and in some studies favourable outcomes were achieved from donors older than 70 years as the transplant community continues to explore an acceptable upper age limit (16–18). In a recent study using the United Network for Organ Sharing (UNOS) database, Haugen *et al.* reported a significantly lower 5-year cumulative mortality rate for patients who accepted livers from donors >70 years when compared to a matched control group who declined the same offer (23% *vs.* 41%) and the authors demonstrated a substantial long-term survival benefit in the former group (19). Despite these favourable outcomes, judicious matching of older donors is paramount as these grafts are more susceptible to ischemia reperfusion injuries, biliary complications, a slight preponderance for hepatic artery thrombosis and the risks are particularly heightened in donors with hepatitis C (17,20,21).

Increasing DCD

Livers from donors with irreversible brain injury, not meeting the criteria for brain death, have resulted in the expansion of the donor pool. A study by Saidi *et al.* reported an increase in the utility of DCD organs from 4.9% to 11.7% during a 10-year study period (22). In the case of LT, recent OPTN data suggests an increase from 4.8% to 6.9% in DCD livers between 2008 and 2018 (12). Several studies comparing DCD livers with standard brain-dead donors (DBD) have demonstrated poorer allograft and patient survival rates in the latter. This is likely related to the longer ischemia time associated with DCD donors, commencing from the time of extubation until cold perfusion, which can be highly variable. A large multicentre study of 2,572 liver transplants comparing DCD and DBD livers identified that 3-year graft loss and recipient mortality were twice as high with DCDs (23). In contrast, a study by Taner *et al.* noted no differences in 1-, 3- and 5-year patient survival rates between DBD and DCD groups and similar comparable outcomes for graft survival (24). In an effort to determine factors leading to graft loss, the authors identified a link between the asystole to cross clamp duration and the development of ischemic cholangiopathy, an important and feared consequence of DCDs. Nonetheless, DCDs can significantly and safely expand the donor pool when used with caution, adhering to a warm ischemia time less than

30 minutes, using donors younger than 50 years and limiting cold ischemia time (7,8). Future efforts to minimize ischemia times and expand the upper age cut-off will further increase the utility of DCD livers and expand the donor pool.

The use of steatotic livers

The rising incidence of obesity and metabolic syndromes has led to the commensurate rise in non-alcoholic fatty liver disease (NAFLD), with a population prevalence of up to 30% in Western societies (25,26). These rising incidences and prevalence rates have seen parallel increases in potential donors with hepatic steatosis. Traditionally, steatotic allografts were avoided in LT for posing significant clinical challenges in terms of early graft dysfunction and PNF. An early study by Spitzer and colleagues analyzing a large US registry of transplant recipients conclusively established that livers with 30% or greater macrovesicular steatosis were associated with lower 1-year survival rates (27). Similar studies have demonstrated unfavourable outcomes in the use of grafts with moderate (30–60%) and severe (>60%) steatosis, largely due to the increased susceptibility of steatotic grafts to ischemia reperfusion injuries (28,29). However, in an effort to further expand the donor pool, fatty livers are increasingly considered for transplantation and form a major component for ECDs. In well matched cases, mild steatosis may have a minimal impact on reperfusion injury and post-transplant hepatic functionality. A study by Dutkowski and colleagues analysed both US and European liver transplant registries and determined grafts with microvesicular and 30% or less macrovesicular steatosis can be used safely with outcomes comparable to non-steatotic livers (30). Acceptable outcomes were also achieved in steatosis greater than 30% with careful risk adjustment, although this remains controversial. Similar studies assessing the suitability of steatotic grafts have also considered the use of moderate to severely steatotic allografts with somewhat acceptable post-transplant outcomes, provided they are supplied by low-risk donors (31,32). Continuing to extend the upper limits of steatosis and define the acceptability of steatotic grafts will still be a matter for debate, however, the integration of these grafts into the donor pool has been a key element in providing more livers for transplant.

Considering donors with an increased infectious risk

The use of donors with exposure to hepatitis B (HBV) or

C (HCV) virus has been more acceptable in current times, albeit previously met with strong objection due to initial concerns regarding the introduction of aggressive viral strains to immunosuppressed recipients. In the context of HCV, there has been a shift in previous attitudes and recent OPTN data reports an increase in the number of livers recovered from hepatitis C positive donors and the number of wait-list candidates willing to accept these livers (12). Rigorous matching remains pertinent and younger donors are preferred due to the fear of increased fibrosis in recipients when older HCV positive donors are used (33). Hepatitis C positive recipients transplanted with positive donors have demonstrated comparable outcomes to those receiving HCV negative livers (34,35). In an effort to further expand the donor pool for all patients, transplanting HCV positive livers into HCV negative patients, with the addition of effective antiviral regimens, has seen some promise in terms of long-term outcomes. Chhatwal and colleagues report compelling evidence demonstrating that accepting HCV positive livers for all donors may increase life expectancy and decrease wait-list mortality (36). Additionally, a recent study by Cotter and colleagues analysing data from 2008 to 2018 reports increased 3-year graft survival rates from 79% to 88% in HCV negative donors receiving HCV positive livers and direct-acting antiviral therapies (37). Similarly, the increased use of organs from HBV positive donors has the potential to expand the donor pool. In a study by Cholongitas and colleagues, recipients without prior exposure to HBV receiving antiviral prophylaxis showed excellent outcomes following transplantation with HBV positive livers (38). Effective HBV prophylaxis in the form of hepatitis B immunoglobulin or oral antiviral therapy have led to a reduction in HBV transmission and viral recurrence in select donors and some units have included these in their donor pool (39–41). While this practice is still limited to a few centres because of the concern of viral reactivation in recipients, more effective prophylaxis could encourage the inclusion of such grafts for the wider recipient pool.

Split liver grafts

Splitting livers (SLT) into two potential grafts has been explored as another method to increase donor supply, however, their use has been relatively stable over the last decade (12). This procedure usually involves donating the left lateral segment to a pediatric recipient and transplanting the right trisegment into an adult, although

splitting the liver into a right and left lobe for two adults has also been described (7,42). It has been estimated that up to 20% of livers are suitable for splitting, however, the technique is limited to specialist centres due to the complexity of the procedure and the heightened risk of biliary and vascular complications (42,43). A study by Vagefi and colleagues assessing outcomes in SLT recipients reported 93%, 77%, and 73% and 89%, 76%, and 65% overall patient and graft survival rates at 1-, 5- and 10-years (44). A similar study by Doyle and colleagues demonstrated 95.5%, 89.5% and 89.5% 1-, 5- and 10-year survival rates in adults recipients of SLT, comparable to whole organ (WLT) recipients (45). In contrast, a multicentre study by Aseni demonstrated lower 5-year survivals in patients receiving SLT compared to WLT (63.3% *vs.* 83.1%) (46). While SLT continues to be limited to centres with technical expertise, they require high quality grafts to gain acceptable post-operative outcomes and currently only represent 1% of US liver transplants and 6% of transplants in Europe (12,47).

Living donor LT (LDLT)

First reported in the US in 1998, LDLT is an additional mechanism to expand the donor pool and increase organ availability. Both right and left lobes can be used in transplantation with the latter generally reserved for small sized recipients and paediatric patients. A left lobe resection has the added benefit of removing a smaller portion of liver from the living donor. By contrast, the right lobe, mainly reserved for adult patients, accounts for up to two-thirds of the total liver mass and can potentially cause significant harm to the donor if the remnant liver volume is too small or unhealthy to support the metabolic and physiological needs of the recipient. The number of LDLTs has increased slowly over the past 2 decades and recent US liver registry data reports LDLTs accounted for up to 4.4% of liver donations in 2018 (12,48). Although modest, the increase in LDLTs has been largely driven by a rise in the number of unrelated directed donors (12). The number of left lobe resections has also decreased corresponding with a parallel rise in right lobe donations. A study by Shah and colleagues demonstrated significantly shorter mean waiting times between right lobe donations and patients awaiting grafts from deceased donors (49). Although LDLT has the advantage of optimizing the timing of surgery, comprehensive donor work-up and minimizing cold ischemia and organ transport times, the process still

poses a risk to the donor and can subject a healthy person to a substantial burden (50). Overall donor mortality rates range from 0.2% to 0.8% with some studies reporting a slight donor survival benefit in right lobe donations (51-53). Graft failure is reported to occur in up to 7.8%, 14.6% and 26.5% of LDLTs at 1, 3 and 5 years with more favourable outcomes demonstrated in non-hepatocellular carcinoma (HCC) diagnoses and Model for End-stage Liver Disease (MELD) scores less than 20 (12). Furthermore, a study by She and colleagues reported, in an analysis of 218 patients, significantly better 5-year graft survival rates in using right lobe LDLT, albeit comparable 5-year patient survival rates (54). These findings were contrasted by Olthoff and colleagues who in a large multicentre study of 963 LDLTs demonstrated similar graft survival rates in right and left lobe LDLTs with decreasing rates of graft failure associated with greater centre experience (55). However, despite these promising results several studies have reported inconsistent graft survival rates between LDLT and standard cadaveric liver donations. Some analyses have demonstrated decreased graft survival rates in LDLT (56,57), while more recent studies appear to demonstrate similar long-term 10-year survival rates between patients receiving grafts from living donors and deceased donor grafts (55). For this reason, LDLT is reserved for high volume centres with specialist expertise due to the complexities of the procedure which requires a deep understanding of the physiological requirements of the graft to ensure sufficient hepatic regeneration and minimize donor risk.

Machine perfusion to optimise liver quality

The recent arrival of *ex-vivo* machine perfusion techniques is beginning to change the landscape of how “marginal” livers are utilized and has the potential to fully expand the donor pool and reduce liver discard. Hypothermic and normothermic machine perfusion technologies have shown promising results in minimising injury to DCD grafts and steatotic livers (58,59). Schlegel and colleagues provided 5-year outcome data on patients receiving DCD liver transplants following treatment with hypothermic oxygen perfusion prior to transplantation. Outcomes of treated DCD livers were similar to DBD and superior to untreated DCDs with significantly less graft loss events (58). A recent study reported similar encouraging results in transplanting previously rejected liver allografts following the application of normothermic machine perfusion (60). While the optimal temperature setting continues to be debated, this

innovation may salvage various extended criteria allografts and modify their risk for routine use, ultimately expanding the donor pool.

Expanding the recipient pool

Despite several strategies attempting to increase the liver donor pool, the recipient pool continues to expand and wait list mortality remains a concern. The wider acceptance of LT and improved outcomes has allowed clinicians to consider transplanting patients they were historically reluctant to do so. According to OPTN data, alcoholic liver disease, non-alcoholic steatohepatitis (NAFLD) and HCC are the most common conditions leading to LT in the US and the increased public burden of these diseases has contributed to an increased number of patients requiring transplantation (12). The healthcare prevalence of alcoholic liver disease continues to grow. A study by Jinjavadia and colleagues observed a substantial increase in the number of alcohol-related hospitalizations over an 8-year period (61). In 2018, the proportion of candidates listed for ALD increased considerably from 22.7% to 29.8% over a 10-year period (12). Similarly, NAFLD is becoming more prevalent and has been linked with the alarming growth rate of population obesity (26). Several models have projected this to become the leading cause for LT as more patients progress to decompensated cirrhosis warranting curative intervention. In addition, global trends have demonstrated a rising incidence in the diagnosis of HCC, although the causes of this are still unclear (62). The evidence regarding transplanting HCC patients, particularly since the introduction of the Milan criteria, has been promising for unresectable disease and more patients are being considered for LT and form a substantial portion of the recipient pool (63,64). The proportion of HCC candidates awaiting LT increased from 3.4% to 9.8% between 2008 and 2018 (12). There has been some interest in expanding the current eligibility guidelines for HCC. Yao and colleagues report similar LT outcomes in HCC patients with tumours as large as 6.5 cm or less than 4 nodules smaller than 4.5 cm and a total tumour diameter less than 8 cm, arguing that the current Milan staging is too restrictive (65). Additionally, the success of chemotherapy has seen several patients with advanced disease downstaged to liver disease within the Milan criteria and meeting eligibility for LT (66). Several other hepatic tumours have been added to the recipient pool with promising results in some units. In a small prospective case-series by Lunsford and colleagues, patients with locally

advanced intrahepatic cholangiocarcinoma and stable disease from neoadjuvant therapy had 1-, 3- and 5-year survival rates of 100%, 83.3%, and 83.3% respectively. While the study only involved a small selected group of patients, LT showed promise (67). In addition, despite initial reluctance, some units have started to reconsider LT for colorectal liver metastases (CLM). In a study comparing LT and chemotherapy for nonresectable CLM, LT observed better 5-year survival rates (56% *vs.* 9%) (68). Although, this has yet to gain widespread acceptance, better expertise in LT and cancer biology could see a rapid expansion in the recipient pool and the inclusion of patients who were previously contraindicated for LT.

Future directions

The transplant community continues to evolve and active efforts to expand both the donor and recipient pools are in constant motion. LT remains a curative option for a wide spectrum of the population and expertise in surgical technique, immunotherapy and post-operative care are among the several elements underpinning the continually improved outcomes reported internationally. While breakthroughs in machine perfusion and chemotherapy are still underway, the role of social media in increasing awareness of organ donation may be a feasible route. Kumar and colleagues, in collaboration with Facebook, created a mobile application whereby waitlisted candidates were allowed the opportunity to create a post and communicate their experience with organ failure and the need for live organ donation (69). Impressively, candidates who engaged with the app were 6.6 times more likely to have a donor come forward compared with matched controls and the app was received well overall (69). As the boundaries of social media platforms continue to push limits, some have advocated for the increased use of social media billboard approaches and targeted campaigns to shift societal perspectives of organ donation and gain public attention (70).

Conclusions

In summary, LT is a growing field with boundless potential. The disparity between patients who need a liver transplant and those who receive one is a constant concern, and while significant progress has been made in the field, further efforts from the national and international consortia are warranted. Pre-existing criteria for LT and recipient boundaries are continually challenged and expanded and,

when coupled with judicious matching and careful patient selection, excellent patient and graft survival results are an endless opportunity.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Mehmet Akce and Shishir K. Maithel) for the series “Hepatocellular Carcinoma” published in *Chinese Clinical Oncology*. The article was sent for external peer review organized by the Guest Editors and the editorial office.

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/cco-20-212>). The series “Hepatocellular Carcinoma” was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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- Cite this article as:** Ahmed O, Doyle MBM. Liver transplantation: expanding the donor and recipient pool. *Chin Clin Oncol* 2021;10(1):6. doi: 10.21037/cco-20-212