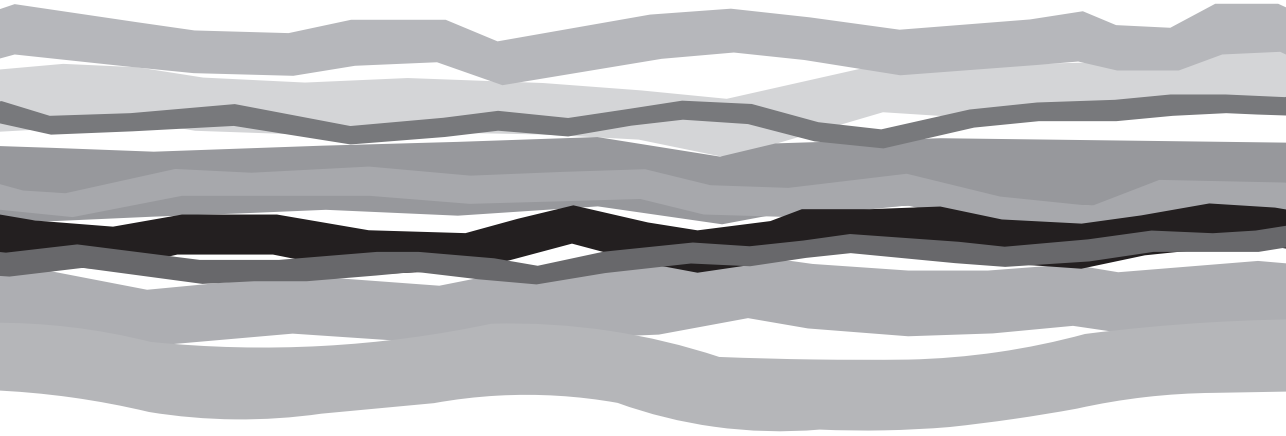
Nutritional and Cellular Preconditioning in Ischemia-reperfusion Injury



Tanja Charlotte Saat

The printing of this thesis has been financially supported by:
Erasmus Universiteit Rotterdam, ChipSoft, Astellas Pharma B.V., Nederlandse Transplantatie Vereniging, Pfizer, Nederlandse Nierstichting

ISBN: 978-94-92683-67-0
Layout and printed by: Optima Grafische Communicatie, Rotterdam, the Netherlands
Cover design by Jim Karstel

Nutritional and Cellular Preconditioning in Ischemia-reperfusion Injury

Nutritionele en cellulaire preconditionering tegen ischemie-reperfusie schade

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

vrijdag 22 september 2017 om 9:30 uur door

Tanja Charlotte Saat

geboren te Rotterdam

Erasmus University Rotterdam

Promotiecommissie:

Promotor:
Prof.dr. J.N.M. IJzermans

Overige leden:
Prof.dr. H.J. Metselaar
Prof.dr. H.G.D. Leuvenink
Prof.dr. C.C. Baan

Copromotor:
Dr. R.W.F. de Bruin

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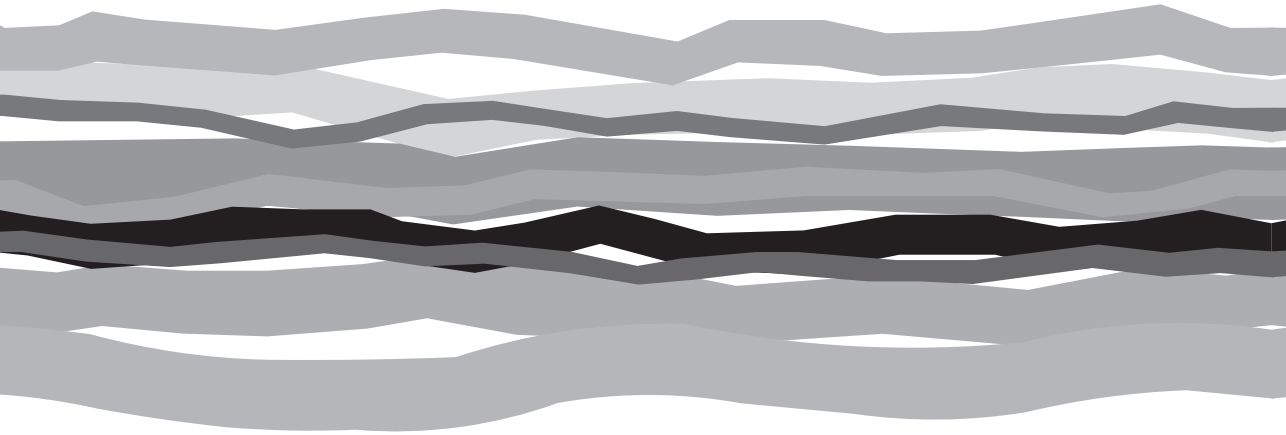
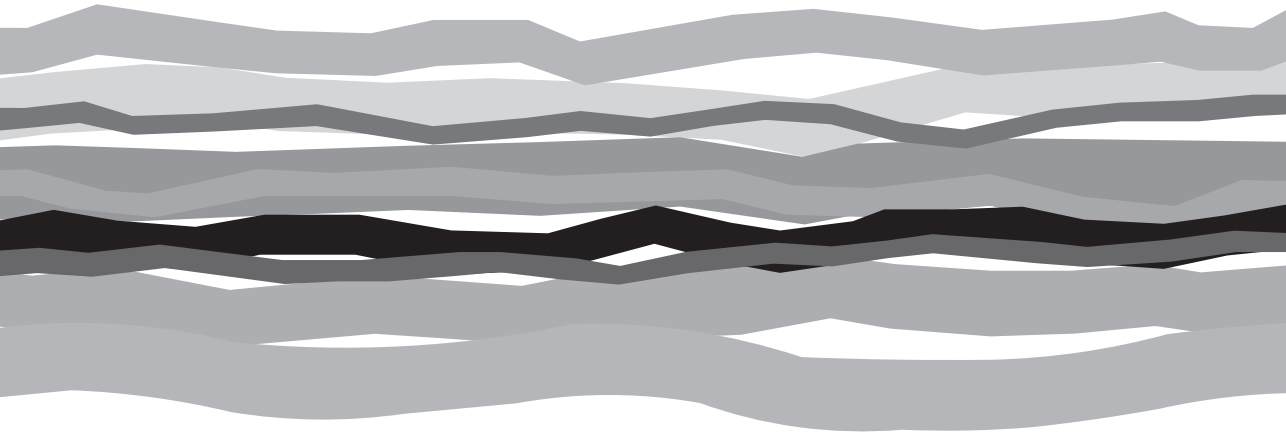


Chapter 1

General introduction and outline of the thesis



General Introduction

Organ transplantation

In the past 60 years, solid organ transplantation is one of the most remarkable and drastic therapeutic achievements in medicine. On December 23, 1954, the first living donor kidney transplantation was performed between identical twins[1,2]. In 1958, successful kidney transplantation between non-identical twins was performed[3]. After the introduction of immunosuppressive drugs the rate of successful kidney transplantation slowly increased during the 1960s[4], with not only living donors but also deceased non-related donors[5]. Since kidney transplantation turned out to be feasible, liver transplantation was developed some years later[6]. In 1967, the first successful liver transplantation was performed in the United States by Thomas Starzl[7].

Improvements in postoperative outcome made organ transplantation a treatment of choice for end-stage organ failure. At present, organ transplantation is a victim of its own success. The improved results have increased the number of patients on the waiting list for organ transplantation, while numbers of deceased donors have remained stable[8]. Eurotransplant statistics showed that in 2016[9], 3278 deceased donor kidneys have been transplanted and 1338 kidneys from living donors. Besides that, 1528 patients received a liver from a deceased donor while 122 patients received a liver from a living donor. However, at the end of 2016, still 10476 patients were waiting for a donor kidney and 1097 patients were waiting for a liver transplant. In an attempt to overcome this discrepancy, not only Donation after Brain Death (DBD) donors are used, but also living donors, Donation after Circulatory Death (DCD) donors and extended criteria donors[10,11].

Living donors are healthy during organ procurement and experience minimal warm and cold ischemia times, which results in superior long-term graft survival compared to DBD- and DCD donors[12]. DBD donors suffer from hemodynamic instability, hormone dysregulation and immunologic reactivity, which contribute to organ damage and affect organ quality[13,14]. Organs from DCD donors are exposed to a period of hypotension, hypoxia and prolonged warm ischemia time. This affects early graft function as well as graft survival[11,15]. Nowadays, the use of extended criteria donors is increasing. Extended criteria donors are defined as donors being >60 years old, or aged >50-59 years old with ≥2 of the following risk factors: history of hypertension, serum creatinine level ≥1.5mg/dL, or death resulting from a cerebrovascular accident[16]. The use of DBD-, DCD- and extended criteria donors, is associated with more severe ischemia reperfusion injury, reducing the organ quality. However, the use of these donors is necessary to overcome the gap between organ demand and supply. The pathophysiology that leads to damage in DBD- and DCD donors organs is a topic of interest for the development of donor specific strategies that might be able to improve post-transplant outcome.

Ischemia reperfusion injury

During organ transplantation and major surgery, ischemia reperfusion injury (IRI) is inevitable and associated with morbidity and mortality[17,18]. Ischemia is caused by an interruption of the blood flow associated with a lack of oxygen and nutrients. This results in a decrease of the oxidative metabolism, depletion of ATP and accumulation of metabolic products[17,19]. Reperfusion leads to rewarming, re-oxygenation, and a return of the aerobic metabolism and the production of ATP. Reperfusion also activates an inflammatory response and reactive oxygen species (ROS) are generated in high concentrations which rise above the capacity of the antioxidant defence, resulting in massive apoptosis[17]. After IRI, repair and regeneration processes occur together with cellular apoptosis, autophagy and necrosis[19].

IRI negatively influences the outcome after transplantation and is a risk factor for primary non-function or delayed graft function. IRI is also associated with acute rejection, and decreased graft and patient survival[17,20]. Many studies were identified to study the mechanisms leading to IRI, and to reduce the impact of IRI on transplant outcome. However, at this moment there is no therapy available to prevent or ameliorate IRI.

Nutritional preconditioning

Dietary restriction (DR) is the reduction in daily calorie and nutrient intake without causing malnutrition[21,22]. A life-long reduction of 30% calorie intake prolongs lifespan, increase health span and improve resistance to multiple stressors in a variety of organisms such as yeasts, worms, fruit flies and rodents[23]. DR was also able to increase lifespan in non-human primates, suggesting DR could also work in humans[24]. Human volunteers on a low-calorie diet showed beneficial changes in their physiological and biochemical parameters[25,26]. The energy metabolism generates ROS that causes molecular oxidative damage and leads to aging[27]. DR decreases the rate of ROS production, and increases the protection against oxidative stress[28]. Therefore, DR is a promising strategy to reduce IRI. Two weeks of 30% DR had a protective effect on IRI in both kidney and liver[29]. In addition, 3 days of fasting induces the same beneficial effects on organ function and improved survival[22,29,30]. The question rises whether the protection of both 2 weeks of 30% DR or 3 days of fasting is based on the reduction in calorie intake or based on a deficiency of a macronutrient (carbohydrates, fat or protein). In fruit flies, long-term protein restriction contributed more to lifespan extension than the reduction in carbohydrates[31]. In fasted mice, glucose supplementation did not interfere with the protection against renal IRI[30]. This point towards a protective essential role for one specific (marco)nutrient. However, long-term restriction of proteins or 30% DR is not achievable in the clinical setting but short-term is. Studies in this thesis focus on the effect of macronutrient- and essential amino acid deficient diets on IRI, the length of time on a diet to induce protective effects and their underlying mechanisms. For implementation in the clinical setting it is

important to unravel the underlying mechanism of nutritional preconditioning to be able to find a mimetic.

Mesenchymal stem cells

Mesenchymal stem cells (MSC) are able to differentiate into cell types other than their tissue of origin, secrete growth factors and have immunomodulatory and anti-inflammatory properties[32,33]. Therefore MSC are considered as a potential therapy in organ transplantation. Last decades, the effect of MSC in small animal models of hepatic IRI or liver regeneration were studied[34-39]. Results show that MSC have anti-apoptotic and anti-inflammatory properties and have the potential to reduce hepatic IRI[35-37]. Moreover, MSC have a beneficial effect on liver regeneration after partial hepatectomy[34,35,38,39]. However, the use of MSC in large animal models of IRI showed inconsistent results[40,41]. It remains unclear if and how MSC are able to prevent IRI and/or stimulate regeneration. Besides that, only few studies investigated the effect of MSCs in a combined hepatic IRI and partial hepatectomy model, while this model is relevant for translation to the clinical setting[39,42,43].

Fibulin-4 deficiency

Previous studies showed that preoperative fasting protects against renal and hepatic IRI in a healthy mouse model. We were curious if preoperative fasting also protect against renal IRI in a mouse model with aortic wall degeneration that might develop aortic aneurysms. Aortic aneurysms are defined as local widening of the artery, several times its normal size, and is a cause of morbidity and mortality in the Western world[44,45]. Rupture leads to extensive internal bleeding and is frequently fatal. Of the abdominal aortic aneurysms 8-20% are proximal abdominal aortic aneurysms (P-AAA), which are characterized by the absence of normal aorta between the upper extent of the aneurysm and the renal arteries[46,47]. During surgical repair of P-AAA the renal arteries are clamped, which causes renal IRI[47,48]. Renal IRI can lead to renal insufficiency, which has been reported as the most frequent complication to suprarenal clamping, and occurs in 22% of the patients[47,49]. Renal insufficiency is a major cause of morbidity, can lead to dialysis and is associated with prolonged hospitalization[50]. Besides that, mortality after surgical P-AAA repair is reported to be 3-8% compared to 1-5% after elective infrarenal abdominal aortic aneurysm repair[47,50]. Standard endovascular repair (EVAR) is feasible in abdominal aortic aneurysms and reduces morbidity and mortality[51]. However, in patients with P-AAA the use of standard EVAR is limited by anatomic restrictions, high costs, and manufacturing lead times[51,52]. Therefore, open surgical repair is still used in patients with P-AAA.

Aortic aneurysms show destructive extracellular matrix remodelling, apoptosis of smooth muscle cells, and inflammatory cell infiltration. Besides gender, age, and life-style-related risk factors, there is evidence that genetic determinants play a role in the

development of aortic aneurysms[53]. Fibulin-4 is an elastic fiber-associated glycoprotein, which plays a role in organization of the extracellular matrix, elasticity and integrity[44,54]. A Fibulin-4 knockout mouse displays embryonic death due to aortic rupture[55]. In a heterozygous Fibulin-4 deficient mouse model, the expression of Fibulin-4 is 2-fold reduced compared to wild type mice. This results in aortic wall degeneration, elastic fiber fragmentation and significantly more local regions of disorganized extracellular matrix and collagen fibers[55]. The heterozygous Fibulin-4 mouse might be a relevant model to learn about the early onset of aortic degeneration and the effect of stressors on blood vessel walls. Since renal IRI is inevitable during surgical repair of P-AAA and treatment of renal IRI is still unsatisfactory, we used this Fibulin-4 deficient mouse model to explore the local and systemic effects of P-AAA repair related renal IRI. Since previous studies showed that preoperative fasting protects against renal IRI[22], we also investigated the effects of preoperative fasting in Fibulin-4 deficient mice.

Aim and outline of the thesis

In the first part we investigated the impact of donor type on transcriptional changes that occur after organ retrieval and cold storage in kidneys and livers from living donors, DCD and DBD donors. Further studies describe the use of nutritional preconditioning and infusion of mesenchymal stem cells as possible treatments to ameliorate IRI. Finally, a mouse model with reduced expression of Fibulin-4 was used to study the local and systemic effects of renal IRI in short- and long-term.

A review of the literature on possible treatment options against renal IRI and the translation from animal experiments towards the clinical setting is described in **chapter 2**.

In **chapter 3**, the impact of living donation, DBD and DCD on transcriptional changes that occur after organ retrieval and cold storage in rat kidneys is investigated. Expression levels of genes indicative of inflammation, cytoprotection, and injury after clinically relevant cold ischemia times were examined. Immunohistochemistry with an early apoptotic marker was performed on DBD- and DCD donor kidneys. In **chapter 4**, the expression levels of inflammatory, cytoprotective and injury genes were examined in rat livers from living donors, DBD and DCD donors. The numbers of apoptotic cells were counted in DBD- and DCD livers after cold preservation.

In **chapter 5**, we investigated in a mouse model whether a preoperative macronutrient free diet protects against renal IRI. We studied food intake, body weight, and kidney function. To unravel the protective mechanism of the diets with a beneficial effect on renal IRI, we created and analysed an unique microarray dataset of different (non-)protective dietary interventions. In **chapter 6**, we studied the protective effect of essential amino acid deficient diets in a mouse hepatic IRI model. Besides phenotypical data, we

used microarray analysis to unravel the specific pathways and/or transcription factors involved in inducing the protective state.

In **chapter 7**, the effect of intravenous infusion of fat derived mesenchymal stem cells in a mouse model of combined hepatic IRI and partial hepatectomy is studied, focussing on liver damage and liver regeneration. In **chapter 8**, a mouse model with reduced expression of Fibulin-4 is used to explore the effect of renal IRI on kidney damage in short- and long-term. We also investigated the effect of preoperative fasting on kidney damage in the heterozygote Fibulin-4 mouse model.

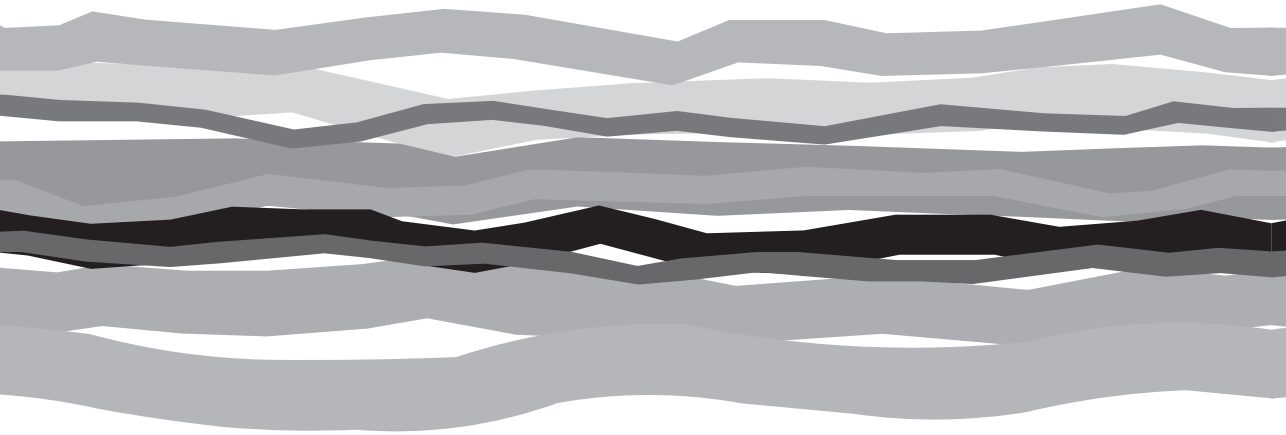
Finally, in **chapter 9**, the findings of this thesis are summarized and discussed, and suggestions are made for future research.

References

1. Murray JE, Merrill JP, Harrison JH. Renal homotransplantation in identical twins. 1955. J Am Soc Nephrol 2001;12:201-4.
2. Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. J Am Med Assoc 1956;160:277-82.
3. Barker CF, Markmann JF. Historical overview of transplantation. Cold Spring Harb Perspect Med 2013;3:a014977.
4. Starzl TE, Marchioro TL, Waddell WR. The Reversal of Rejection in Human Renal Homografts with Subsequent Development of Homograft Tolerance. Surg Gynecol Obstet 1963;117:385-95.
5. Sayegh MH, Carpenter CB. Transplantation 50 years later--progress, challenges, and promises. N Engl J Med 2004;351:2761-6.
6. Linden PK. History of solid organ transplantation and organ donation. Crit Care Clin 2009;25:165-84, ix.
7. Saidi RF. Current status of liver transplantation. Arch Iran Med 2012;15:772-6.
8. Lee J. Critical shortage. As technology improves, the transplant list gets longer--but there aren't enough organs to go around. Mod Healthc 2013;43:6-7, 1.
9. Eurotransplant. Organs transplanted in 2015. 2015.
10. Taner CB, Bulatao IG, Willingham DL, et al. Events in procurement as risk factors for ischemic cholangiopathy in liver transplantation using donation after cardiac death donors. Liver Transpl 2012;18:100-11.
11. Weber M, Dindo D, Demartines N, Ambuhl PM, Clavien PA. Kidney transplantation from donors without a heartbeat. N Engl J Med 2002;347:248-55.
12. Chkhotua AB, Klein T, Shabtai E, et al. Kidney transplantation from living-unrelated donors: comparison of outcome with living-related and cadaveric transplants under current immunosuppressive protocols. Urology 2003;62:1002-6.
13. Barklin A. Systemic inflammation in the brain-dead organ donor. Acta Anaesthesiol Scand 2009;53:425-35.
14. Floerchinger B, Oberhuber R, Tullius SG. Effects of brain death on organ quality and transplant outcome. Transplant Rev (Orlando) 2012;26:54-9.
15. Monbaliu D, Pirenne J, Talbot D. Liver transplantation using Donation after Cardiac Death donors. J Hepatol 2012;56:474-85.
16. Pascual J, Zamora J, Pirsch JD. A systematic review of kidney transplantation from expanded criteria donors. Am J Kidney Dis 2008;52:553-86.
17. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004;364:1814-27.
18. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. World J Gastroenterol 2013;19:1683-98.
19. Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 2008;40:3279-88.
20. Uemura T, Randall HB, Sanchez EQ, et al. Liver retransplantation for primary nonfunction: analysis of a 20-year single-center experience. Liver Transpl 2007;13:227-33.
21. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition 1989;5:155-71; discussion 72.
22. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010;9:40-53.

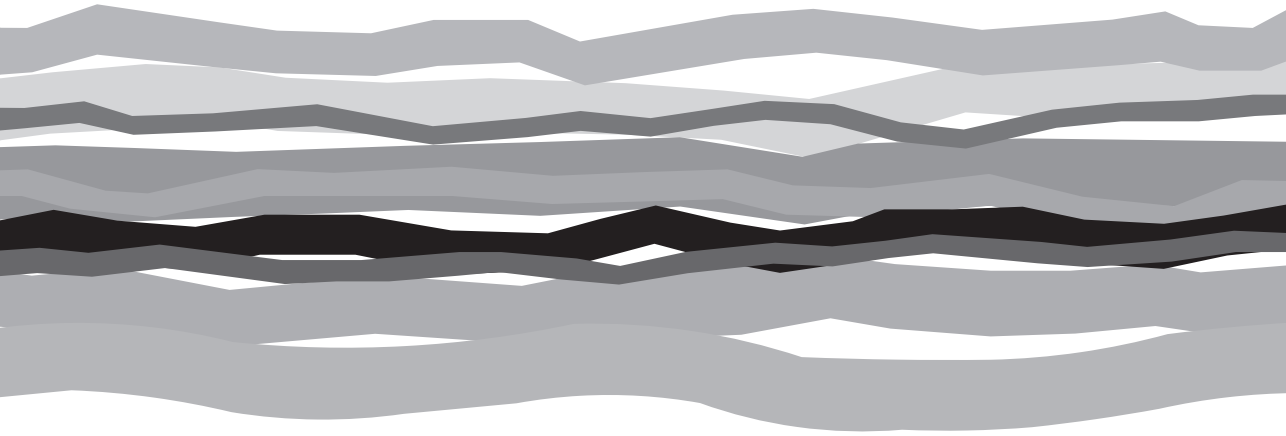
23. Masoro EJ. Subfield history: caloric restriction, slowing aging, and extending life. Sci Aging Knowledge Environ 2003;2003:RE2.
24. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009;325:201-4.
25. Walford RL, Mock D, Verdery R, MacCallum T. Calorie restriction in biosphere 2: alterations in physiologic, hematologic, hormonal, and biochemical parameters in humans restricted for a 2-year period. J Gerontol A Biol Sci Med Sci 2002;57:B211-24.
26. Heilbronn LK, de Jonge L, Frisard MI, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. Jama 2006;295:1539-48.
27. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956;11:298-300.
28. Hine CM, Mitchell JR. NRF2 and the Phase II Response in Acute Stress Resistance Induced by Dietary Restriction. J Clin Exp Pathol 2012;S4.
29. Verweij M, van Ginhoven TM, Mitchell JR, et al. Preoperative fasting protects mice against hepatic ischemia/reperfusion injury: mechanisms and effects on liver regeneration. Liver Transpl 2011;17:695-704.
30. Verweij M, van de Ven M, Mitchell JR, et al. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation 2011;92:752-8.
31. Mair W, Piper MD, Partridge L. Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS Biol 2005;3:e223.
32. Souidi N, Stolk M, Seifert M. Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells. Curr Opin Organ Transplant 2013;18:34-43.
33. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal Stem Cells Induce an Inflammatory Response After Intravenous Infusion. Stem Cells Dev 2013.
34. Li DL, He XH, Zhang SA, Fang J, Chen FS, Fan JJ. Bone marrow-derived mesenchymal stem cells promote hepatic regeneration after partial hepatectomy in rats. Pathobiology 2013;80:228-34.
35. Kanazawa H, Fujimoto Y, Teratani T, et al. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. PLoS One 2011;6:e19195.
36. Jin G, Qiu G, Wu D, et al. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. Int J Mol Med 2013;31:1395-401.
37. Sun CK, Chang CL, Lin YC, et al. Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats. Crit Care Med 2012;40:1279-90.
38. Kaibori M, Adachi Y, Shimo T, et al. Stimulation of liver regeneration after hepatectomy in mice by injection of bone marrow mesenchymal stem cells via the portal vein. Transplant Proc 2012;44:1107-9.
39. Seki T, Yokoyama Y, Nagasaki H, Kokuryo T, Nagino M. Adipose tissue-derived mesenchymal stem cell transplantation promotes hepatic regeneration after hepatic ischemia-reperfusion and subsequent hepatectomy in rats. J Surg Res 2012;178:63-70.
40. Behr L, Hekmati M, Lucchini A, et al. Evaluation of the effect of autologous mesenchymal stem cell injection in a large-animal model of bilateral kidney ischaemia reperfusion injury. Cell Prolif 2009;42:284-97.

41. Behr L, Hekmati M, Fromont G, et al. Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 2007;107:p65-76.
42. Wang W, Du Z, Yan J, et al. Mesenchymal stem cells promote liver regeneration and prolong survival in small-for-size liver grafts: involvement of C-Jun N-terminal kinase, cyclin D1, and NF-kappaB. PLoS One 2014;9:e112532.
43. Saidi RF, Rajeshkumar B, Shariftabrizi A, et al. Human adipose-derived mesenchymal stem cells attenuate liver ischemia-reperfusion injury and promote liver regeneration. Surgery 2014;156:1225-31.
44. Kaijzel EL, van Heijningen PM, Wielopolski PA, et al. Multimodality imaging reveals a gradual increase in matrix metalloproteinase activity at aneurysmal lesions in live fibulin-4 mice. Circ Cardiovasc Imaging 2010;3:567-77.
45. Lindsay ME, Dietz HC. Lessons on the pathogenesis of aneurysm from heritable conditions. Nature 2011;473:308-16.
46. Johnston KW, Rutherford RB, Tilson MD, Shah DM, Hollier L, Stanley JC. Suggested standards for reporting on arterial aneurysms. Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular Surgery. J Vasc Surg 1991;13:452-8.
47. West CA, Noel AA, Bower TC, et al. Factors affecting outcomes of open surgical repair of pararenal aortic aneurysms: a 10-year experience. J Vasc Surg 2006;43:921-7; discussion 7-8.
48. Dubois L, Durant C, Harrington DM, Forbes TL, Derose G, Harris JR. Technical factors are strongest predictors of postoperative renal dysfunction after open transperitoneal juxtarenal abdominal aortic aneurysm repair. J Vasc Surg 2013;57:648-54.
49. Le Dorze M, Legrand M, Payen D, Ince C. The role of the microcirculation in acute kidney injury. Curr Opin Crit Care 2009;15:503-8.
50. Kabbani LS, West CA, Viau D, et al. Survival after repair of pararenal and paravisceral abdominal aortic aneurysms. J Vasc Surg 2014;59:1488-94.
51. Banno H, Cochennec F, Marzelle J, Becquemin JP. Comparison of fenestrated endovascular aneurysm repair and chimney graft techniques for pararenal aortic aneurysm. J Vasc Surg 2014;60:31-9.
52. Jongkind V, Yeung KK, Akkersdijk GJ, et al. Juxtarenal aortic aneurysm repair. J Vasc Surg 2010;52:760-7.
53. Davis FM, Rateri DL, Daugherty A. Mechanisms of aortic aneurysm formation: translating preclinical studies into clinical therapies. Heart 2014.
54. Chen Q, Zhang T, Roshetsky JF, et al. Fibulin-4 regulates expression of the tropoelastin gene and consequent elastic-fibre formation by human fibroblasts. Biochem J 2009;423:79-89.
55. Hanada K, Vermeij M, Garinis GA, et al. Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. Circ Res 2007;100:738-46.



Chapter 2

Improving the outcome of kidney transplantation by ameliorating renal ischemia reperfusion injury: Lost in translation?



Tanja C. Saat, Eline K. van den Akker, Jan N.M. IJzermans,
Frank J.M.F. Dor, Ron W.F. de Bruin

Journal of Translational Medicine

Abstract

Kidney transplantation is the treatment of choice in patients with end stage renal disease. During kidney transplantation ischemia reperfusion injury (IRI) occurs, which is a risk factor for acute kidney injury, delayed graft function and acute and chronic rejection. Kidneys from living donors show a superior short- and long-term graft survival compared with deceased donors. However, the shortage of donor kidneys has resulted in expansion of the donor pool by using not only living- and brain death donors but also kidneys from donation after cardiac death and from extended criteria donors. These grafts are associated with an increased sensitivity to IRI and decreased graft outcome due to prolonged ischemia and donor comorbidity. Therefore, preventing or ameliorating IRI may improve graft survival. Animal experiments focus on understanding the mechanism behind IRI and try to find methods to minimize IRI either before, during or after ischemia. This review evaluates the different experimental strategies that have been investigated to prevent or ameliorate renal IRI. In addition, we review the current state of translation to the clinical setting. Experimental research has contributed to the development of strategies to prevent or ameliorate IRI, but promising results in animal studies have not yet been successfully translated to clinical use.

Introduction

Kidney transplantation is the treatment of choice in patients with end stage renal disease. Increased prevalence of end stage renal disease, and improved results after kidney transplantation have contributed to the increased shortage of donor organs and the need to expand the donor pool[1,2]. Organs from living donors have a superior graft survival compared with deceased donors[3,4]. The superior outcome of living donor kidneys is associated with shorter warm and cold ischemia, shorter waiting time for the recipient and 'healthier' donor kidneys[5]. Warm ischemia occurs after the blood supply has been cut off while the organ is still in the donor. During storage of the donor organ, the temperature is reduced to approximately 4°C. During this cold ischemia period, metabolism is significantly reduced which allows for prolonged preservation of the organ until transplantation.

To bridge the growing gap between organ demand and supply, Donation after Circulatory Death (DCD) donors[6] and extended criteria donors are increasingly being used[3-5]. Donation after Brain Death (DBD) donors are exposed to physiological changes during brain death, which may lead to organ damage and inferior graft survival compared to living donors[7,8]. DCD donors do not develop the physiological changes of DBD donors, but suffer from prolonged warm ischemia times during cardiac arrest. DCD kidneys have an increased incidence of delayed graft function (DGF) of 73% compared to 27% in DBD kidneys[9], while the rate of acute rejection is similar in both. Despite the higher incidence of DGF, DCD kidneys show no differences in long-term graft survival compared with DBD kidneys[6,10].

Although the use of DCD donors has been increased, the total number of cadaveric donors remains stable, while the waiting list continues to grow[6,11]. Therefore, the number of extended criteria donors is increasing. Extended criteria donors are defined as donors being >60 years old, or aged >50-59 years old with ≥2 of the following risk factors: history of hypertension, serum creatinine level ≥1.5 mg/dL, or death resulting from a cerebrovascular accident[12,13]. Organs from extended criteria donors are associated with a higher incidence of DGF, lower graft survival and suboptimal kidney function[13,14]. Recipients of kidneys procured from extended criteria donors show an 1.7 fold greater risk of graft lost compared to recipients with a kidney from an 'ideal donor' (10-39 years old without hypertension or stroke as a cause of death and a serum creatinine concentration <1.5mg/dL)[15].

Ischemia reperfusion injury (IRI) is an inevitable consequence of kidney transplantation and has major consequences for graft- and patient survival[16-18]. Renal IRI is a known risk factor for DGF[19], acute kidney injury[20] and acute and chronic rejection[21]. Donor type is strongly associated with the severity of renal IRI[22]. DCD donors and extended criteria donors are more vulnerable to IRI since donor kidneys suffer from prolonged warm isch-

emia time, increased donor age or comorbidity of the donor[13,14]. Prevention or reduction of IRI could improve graft survival and decrease patient morbidity.

Renal ischemia reperfusion injury

Renal IRI is unavoidable during transplantation and is a risk factor for DGF[19], acute kidney injury[18,23] and acute and chronic rejection[24,25]. Acute kidney injury is associated with high morbidity, prolonged hospitalization, and increased mortality[20,26]. During ischemia there is a lack of O_2 and nutrients, which results in a decrease of oxidative metabolism, accumulation of metabolic waste products and depletion of ATP[19,20]. Reperfusion leads to rewarming, re-oxygenation and a return to aerobic metabolism. However, reactive oxygen species are generated which directly injure the cytoskeletal and functional cellular components[19]. Normally, antioxidant enzymes may counteract the effects of reactive oxygen species, but their protective effect is overwhelmed by the rapid production of reactive oxygen species, resulting in tissue injury and cell death[27]. During reperfusion, tissue injury is exacerbated by an inflammatory response, which initiates a cascade of deleterious cellular responses[18,19]. Inflammatory cytokines are up-regulated, and chemokines and complement are released, which results in the migration and activation of leukocytes. The mechanism underlying IRI is multifactorial. Due to its complexity, IRI provides different targets to prevent or ameliorate renal IRI before, during or after transplantation[23].

Strategies to ameliorate renal IRI

Strategies to reduce renal IRI can be implemented in both donor and recipient, and before, during and after transplantation. Treatment of IRI can be focused on scavenging reactive oxygen species, reducing inflammation, stimulating cell survival and regeneration, or a combination thereof. Prevention of ischemia is impossible by inducing resistance against ischemia before organ retrieval. Pre-treatment of living donors is feasible, provided it does not affect the health and wellbeing of the donor. In post-mortem donors the situation is more difficult since these donors are not able to give informed consent and ethical issues may rise. During preservation treatment is possible by using machine preservation and/or by adding protective agents to the perfusion fluid, pre- or post-conditioning is feasible during transplantation. After transplantation, treating the recipient, after informed consent, may reduce the damage caused by IRI. In this review, we focus on experimental and clinical studies on dietary preconditioning, preservation, ischemic pre- and post-conditioning, cell therapy, pharmacological treatment and microRNAs as intervention strategies to reduce renal IRI. In addition, we review the current state of translation to the clinical setting of these interventions (**Figure 1**).

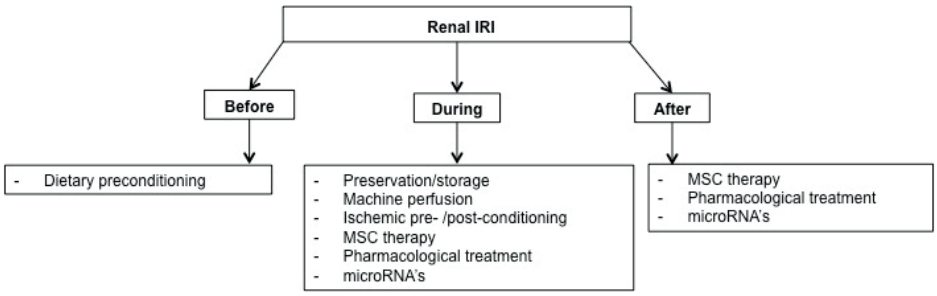


Figure 1. Overview of various therapies before-, during- and after kidney transplantation, which are capable of ameliorating renal ischemia reperfusion injury in animal models.

Dietary preconditioning

Dietary restriction is a reduction in food intake without malnutrition, and is associated with extended life span, improved metabolic fitness and increased resistance to oxidative stress in a wide range of organisms[28-30]. In mice, short-term 30% dietary restriction or 3 days of fasting, reduced kidney damage and dysfunction and improved survival after renal IRI[30]. Short-term dietary restriction and fasting increased expression of cytoprotective genes and decreased the expression of inflammatory markers[30]. Food restriction leads to a reduction in both calorie and nutrient intake, yet the contribution of calories or nutrients to the protective effect on renal IRI is unknown[28]. Verweij et al.[31] showed that the benefits of preoperative fasting are not affected by the intake of calories via glucose water during fasting from solid food. Subsequently, diets lacking protein or even the essential amino acid tryptophan for 6-14 days resulted in similar protection against renal IRI in mice[32-34]. Therefore, a preoperative calorie restricted diet might be a non-invasive way to reduce IRI after human kidney transplantation. Although the beneficial effects of a preoperative diet are in apparent conflict with the patients' nutritional wellbeing[35,36], several recent clinical studies showed that the human response to dietary restriction is similar to that observed in experimental mammalian models[37,38]. In human living kidney donors short-term dietary restriction before surgery is feasible, well tolerated and safe[39], although the conditions to induce a similar powerful protection against IRI as in mice have not been elucidated yet. More clinical research is needed to translate the beneficial effect of preoperative diets from animals to humans.

Preservation

Another option to decrease IRI is to minimize damage caused during ischemia. Prolonged cold ischemia time has a strong association with development of DGF[19,25], but a decrease in cold ischemia time is difficult due to logistics, allocation and organ transport. Optimisation of conditions during cold ischemia time is therefore essential.

Cold storage solutions were designed to increase organ tolerance and preserve cellular integrity during ischemia[40,41]. Reducing the temperature of the kidney to 4˚C reduces enzyme activity, decreases oxygen requirement and lowers metabolism by 58%[42]. Some studies add nutrients or pharmacologically active agents to the preservation solution (reviewed in Chatauret et al.[43]). Cold storage is still considered the gold standard in kidney preservation.

During machine perfusion, the organ is attached to a machine during preservation, which pumps preservation solution through the organ. It creates the possibility to maintain hemodynamic stimulation, administer nutrients to the kidney and even eliminate toxins. A large international prospective randomized controlled trial The Netherlands showed the benefits of machine perfusion by reducing the incidence and duration of DGF, in DCD kidneys[44]. Also, machine perfusion of extended criteria donor kidneys reduced the rate of DGF[45,46], is feasible and safe[47]. In a meta-analysis, Deng et al.[48] compared the transplant outcomes in patients receiving DCD kidneys preserved by machine perfusion or by static cold storage. Recipients with a DCD kidney preserved by machine perfusion had a decreased incidence of DGF compared to static cold storage. However, there is no significant difference between the two groups in incidence of primary non-function, graft survival or patient survival after one year.

Hypothermic machine preservation slows down the metabolism of the kidney and allows an organ to be stored without oxygen for a short period of time but this process also causes cellular damage. Therapeutic agents have been added to the preservation solution during hypothermic machine preservation but the hypothermic conditions make it difficult for the agent while the metabolism is blocked. Maintaining the kidney at a normothermic temperature has many advantages. The kidney is able to regain function and can minimize the cold ischemia time. The kidney can be maintained in a stable state and it provides the opportunity to add therapeutical agents to a functioning organ[49]. Machine perfusion is one of the therapeutic interventions that is making the translation to humans. Randomized controlled trials are now being developed and will guide machine perfusion into the clinical arena.

Ischemic Pre-/Post-conditioning

Ischemic conditioning is defined as applying a brief ischemic insult to an organ through brief (repetitive) sequences of ischemia and reperfusion before or after an ischemic attack to provide resistance against IRI. Ischemic preconditioning (IPC) and ischemic post-conditioning (IPoC) were both developed in cardiac research, but may be applied in the kidney as well, reviewed in Kierulf-Lassen et al.[50]

Ischemic pre-conditioning

In 1986, protection against IRI by IPC was first seen in canine hearts[51]. Dogs were pre-conditioned with four repetitive sequences of ischemia and reperfusion each 5 minutes, followed by 40 minutes of occlusion. IPC limited infarct size to 25% compared to the control group. After these findings many animal experiments have been done to reproduce this protective effect in other organs[52]. In the kidney, IPC induces improved renal function and histology after transplantation[53].

In remote IPC, the ischemic trigger is not applied locally to the target organ, but on another 'remote' organ[54]. Patients undergoing elective coronary artery bypass graft surgery underwent remote IPC consisting of three 5-minute cycles of right upper limb ischemia directly after anesthesia. Remote IPC reduced serum troponin-T release compared to patients undergoing coronary artery bypass graft surgery without IPC[55].

In a rat model, renal IRI was induced by a right nephrectomy and clamping the left renal artery for 60 minutes. Remote IPC was induced by 5-minute cycles of ischemia and reperfusion, occluding the right hind limb. The remote IPC groups showed lower levels of kidney dysfunction and damage[56]. In a renal IRI pig model, remote IPC was induced by clamping the left iliac artery for 10 minutes, which showed no beneficial effects on renal function or histology[57]. In humans, kidney transplant recipients underwent remote IPC and were compared to paired recipients without IPC. Remote IPC was induced by three cycles of 5 minutes of brief repetitive ischemia by clamping the exposed external iliac artery. Serum creatinine levels were lower in the remote IPC group, while glomerular filtration rates were higher during the first 14 days post-transplant. These results suggest that remote IPC has beneficial effects on the early recovery of renal function after kidney transplantation[58].

Remote preconditioning is a potential therapeutic strategy that can reduce renal IRI, and is simple to apply, non-invasive and virtually cost-free, but large multi-center clinical trials using remote IPC are needed to improve the level of evidence and implement remote IPC in the clinical setting. Results of a large international prospective randomized controlled trial (CONTEXT trial) are eagerly awaited[59].

Ischemic post-conditioning

IPoC, defined as rapid, intermittent interruptions of blood flow at the onset of reperfusion can reduce myocardial infarct size in animal models[60,61]. The use of IPoC in humans undergoing cardiac surgery showed better post-operative outcomes[62]. Similar beneficial effects have also been observed in animal models of renal IRI[63]. IPoC reduced tubular necrosis after reperfusion, and attenuated renal dysfunction[64]. Its observed benefits are associated with an enhanced expression level of SOD and inhibition of apoptosis[65]. These effects are seen in different animal species with different index ischemia times and different algorithms. Only two studies did not observe a significant difference in

renal function, which could be explained by the time points of analyzing renal function, which were either too early (2 hours) or too late (12 weeks) after reperfusion[66,67].

Contrary to IPoC, remote IPoc has only been performed in 2 renal IRI rat studies[56,68]. Remote IPoC of the hind limb resulted in significant improvement in renal function 24 hours after IRI. Sequences of ischemia and reperfusion during the ischemic episode, PER-conditioning, was able to reduce renal IRI even further[68]. As with IPC, the first attempts to translate IPoC into human kidney transplantation are already being done[69]. Unfortunately, the robust beneficial effects as seen in animal experiments, have not been observed yet. IPoC is feasible and safe in patients undergoing kidney transplantation, but the proper algorithm that reduces the incidence of DGF still has to be found[69].

Cellular therapy

Administration of cells to modulate the course of IRI has attracted considerable interest. Two cell types in particular, mesenchymal stem cells (MSC), and regulatory T cells (Tregs) have been investigated.

MSC are able to differentiate into cell types other than their tissue of origin, are non-immunogenic, immunosuppressive, able to migrate, secrete growth factors and anti-apoptotic cytokines[70], and might play a role in tissue repair. Due to these characteristics, MSC are promising as a cell therapy to reduce renal IRI. In rodent renal IRI models, MSC were able to up-regulate the cytoprotective genes HO-1 and SOD[71-73], reduce oxidative stress and apoptosis[71], and improve kidney function[71,72,74]. Furthermore, kidneys treated with MSC showed a stronger regenerative response[75].

Subsequently, in large-animal models, MSC failed to reduce cell death and no changes in proliferation or cytokine release were found[76,77]. It might be that the optimal time window for stem cell therapy is different in large-animal models than in rodents. Another problem is poor cell survival of injected MSC. After intravenous injection MSC home to the lungs and within 24 hours the majority of MSC die, MSC do not migrate to the site of injury and do not contribute to structural renal repair[78]. This suggests that the effect of the MSC might result from paracrine or endocrine effects unrelated to their differentiation capacity[78-82].

Early clinical trials have attempted to translate the potential immunosuppressive effects of MSC, but results were not convincing. Perico et al.[83] were the first to report on two patients undergoing living kidney transplantation and receiving an infusion of autologous MSC on post-transplant day 7. Serum creatinine levels were increased in MSC-treated patients 7 to 14 days after infusion, suggesting dysfunction of the graft. One year post-transplantation kidney biopsies showed no signs of rejection. Their conclusion was that MSC therapy in kidney transplantation is feasible, although timing, doses and immunosuppressive medication may need to be adapted for optimal effect. Reinders et al.[84] studied the feasibility of autologous MSC administration in kidney transplanta-

tion recipients and showed it to be feasible and safe, although the study does not allow conclusions on efficacy. Peng et al.[85] combined MSC with a sparing dose of Tacrolimus (50% of standard dose) in living-related kidney transplant recipients. Patients received two infusions of MSC, the first directly into the renal artery at the time of transplantation, the second intravenously one month later. Results suggest that MSC therapy is safe and could reduce the dosage of Tacrolimus. The results of both animal models and clinical trials are encouraging, but the low number of randomized controlled trials and small numbers of patients make it difficult to draw definitive conclusions and implement MSC therapy in transplantation[86].

The knowledge that Tregs have a crucial role in control of autoimmunity and tolerance induction in transplantation has made the induction of-, or infusion of Tregs a possible treatment for an array of inflammatory conditions, IRI. In humans, intravenous infusion of Tregs is not only feasible and safe, but reduced the incidence of graft versus host disease in patients with hematologic malignancy that were treated with stem cell transplantation[87]. Furthermore, after inducing renal IRI in rodents, Tregs are able to suppress renal inflammation and preserve renal function[88]. In a mouse model, Treg deficiency resulted in enhanced renal inflammation, acute tubular necrosis and loss of function. Suppletion of Tregs protected mice from renal dysfunction and improved survival[89]. Although the use of Tregs as a cellular therapy against renal IRI seems promising, studies in humans with renal IRI are lacking. The use of Tregs in humans is troubled by numerous challenges. The dose of Tregs needed for therapeutic efficacy is unclear, the isolation of pure Tregs is difficult due to the absence of Treg-specific cell surface markers and safety is still a topic of concern[90].

Pharmacological treatment

Although many pharmacological agents are effective in experimental models of IRI and acute kidney injury, none of these have successfully been implemented in standard clinical care protocols. With few exceptions, most do not enter the clinic. An overview of tested pharmacological substances is given by Bajwa et al.[91]. Of the eight substances that might reduce inflammation and reduce cytotoxicity they focused on, only two were tested in clinical studies for acute kidney injury (statins and erythropoietin). However, in human studies the results on renal IRI induced acute kidney injury are conflicting. Retrospective case controlled studies found that statins reduced acute kidney injury in patients with contrast-induced nephropathy[92,93], whereas a prospective study did not find any beneficial effects[94]. Remarkably, a number of observational studies suggested that in the first few weeks and months of starting a statin, statins were associated with the early development of acute kidney[95]. Due to the adverse data and the lack of good prospective randomized controlled trials, there is no evidence that statins reduce the incidence of acute kidney injury.

Preconditioning with erythropoietin protects against IRI in rodents[96,97]. Encouraged by these results erythropoietin was injected intravenously in humans before surgery, and was able to reduce the incidence of acute kidney injury in patients who underwent a coronary artery bypass[98]. Xin et al.[99] published a meta-analysis including four randomized controlled trials that investigated high-dose erythropoietin on graft function after kidney transplantation. The results showed that high-dose erythropoietin is able to reduce the number of patients with DGF, but these results did not reach significance. However, Vlachopanos et al.[100] published a meta-analysis to explore the impact of recombinant human erythropoietin on DGF in kidneys from deceased donors. Four randomized controlled trials were included and perioperative high-dose recombinant human erythropoietin was compared with placebo or no therapy. High-dose recombinant human erythropoietin did not affect mortality, acute rejection, DGF or kidney function 4 weeks after transplantation. Remarkably, the systolic blood pressure was significantly higher in patients treated with recombinant human erythropoietin. These results question the efficacy and safety of high-dose human recombinant erythropoietin in humans. Despite the promising results in animal models, translating these findings to the clinic is difficult. Variable factors as dosage and time points of injection might be a topic of interest for further clinical trials.

Recently, nonerythropoietic peptides derived from the three-dimensional structure of erythropoietin were shown to exert tissue protective properties. It was shown that the helix B surface peptide of erythropoietin is responsible for the tissue protective effect of erythropoietin and has a much better stability[101]. In a mouse renal IRI model, helix B peptide improved renal function, decreased apoptosis, inflammation and histological injury[102]. Yang et al.[103] added the helix B peptide to preservation and reperfusion solutions used to normothermically perfuse porcine kidneys after 20 minutes of warm ischemia and 18 hours of cold ischemia. Adding helix B peptide to the reperfusion solution improved the renal blood flow, oxygen consumption and urine output during reperfusion and decreased renal tissue damage. Helix B peptide could be the key needed to translate the beneficial effects of erythropoietin to human transplantation.

MicroRNA's

MicroRNA's are RNA-molecules of 20-25 nucleotides long. They are capable to inhibit protein transcription by stimulating degradation of mRNA[104]. The majority of gene expression is regulated in this way. A promising quality of microRNA's is their stability in body fluids[105], which makes them a good candidate to act as a biomarker or as a therapeutical target. One microRNA can inhibit more than 100 genes, so determining the role of microRNAs in IRI is difficult. The few studies on microRNAs in renal IRI failed to elucidate an unequivocal microRNA-signature[106-108]. Expression profiling of microRNAs following renal IRI in a mouse model showed that nine miRNAs (miR-21, miR-20a, miR-146a, miR-

199a-3p, miR-214, miR-192, miR-187, miR-805, and miR-194) are differently expressed compared to sham animals[108]. In vitro studies revealed that miR-21 is expressed in proliferating tubular epithelial cells, and overexpression of miR-21 has a protective effect against cell death. This might suggest that miR-21 plays a role in protection against IRI. In humans, microRNA expression profiles have been analysed to see if microRNAs may predict the outcome after kidney transplantation[106,107]. In renal biopsies of patients with acute rejection, 20 differentially expressed miRNAs were identified[106]. These expression profiles may provide useful information about the outcome after kidney transplantation. Unfortunately, research so far has not brought major insights in the role of microRNAs as therapeutic target or agent in both animals and humans[109].

Discussion

The improved results after kidney transplantation and the increased waiting list have contributed to the growing gap between organ demand and supply. Extension of the donor pool is needed to diminish this gap. Therefore, there has been a shift to the use of DCD donors and extended criteria donors. DCD donors have an increased incidence of DGF compared to DBD donors, while the rate of acute rejection is similar in both groups[9,10]. Kidneys from extended criteria donors have a higher risk of DGF, lower graft survival and suboptimal kidney function[13,14]. Although renal IRI is inevitable during transplantation and has detrimental effects on the outcome, there is no therapy available. Therefore, finding a method to ameliorate renal IRI is of major interest.

Renal IRI can be treated before-, during-, and after- transplantation, or a combination thereof. When treatment is applied before ischemia, translation of these data is difficult since in the human setting, treatment before ischemia would imply treatment of the donor. This raises ethical concerns in DCD donors[110]. During treatment of the living donors, the donor must give full consent and treatment should not interfere with the donor's health. An option for treatment of the (living) donor before transplantation is dietary preconditioning. Van Ginhoven et al.[39] showed that dietary pretreatment of living donors is feasible and safe, but the robust effects on reducing IRI as observed in mice are lacking. Despite many experimental studies which show beneficial effects on an array of treatments and interventions against IRI, translation to humans has not been successful[111].

In animal experiments, genetic variability is low and mostly young, healthy, males are used. This is obviously not representative for the population that is undergoing kidney transplantation and is experiencing renal IRI. Overweight, comorbidities, old age, gender and the use of medication can all interfere with the effects of studied methods to ameliorate renal IRI[112,113]. Another limitation of animal experiments may be the use of

warm ischemia models to mimic transplantation induced IRI[30,31,71,74]. The use of cellular therapy is difficult to translate to humans due to the differences between animals and humans. More clinical trials are needed to evaluate the effect of both MSC and Tregs. It would be of tremendous value to use MSC, or to be able to induce the production of Tregs in the recipient to ameliorate renal IRI.

Besides these translational difficulties, another problem in the treatment of IRI is its pathophysiological complexity. Many pathological mechanisms contribute to IRI and can be focused on. Studies on IRI treatment are therefore divers, and the probability to find a single therapeutical agent is low. Besides that, experimental therapeutical agents may induce adverse side effects[114-117] or be carcinogenic[118,119] which limits their use in humans. Another difficulty in translating animal results into humans is the publication bias. It is difficult to get an objective overview of the results of experimental therapies since there may be a bias towards publication of studies with a positive outcome[120].

Nevertheless, machine perfusion and (remote) ischemic pre- and post-conditioning are promising treatment options, which are feasible and safe. Especially machine perfusion induces beneficial effects on kidney function after transplantation in various donor types, and large randomized controlled trials are being conducted. The use of machine perfusion is actually making the translation to the clinical arena.

Renal IRI is a highly relevant detrimental consequence of kidney transplantation and therefore an important topic in transplantation research. Studying renal IRI is complex though, coping with translational difficulties, and multifactorial pathophysiological mechanisms. Although animal studies have resulted in promising methods to ameliorate renal IRI, we are still lost in translation since only few animal data are finding their way into the clinic and improve transplant outcome. This gap in our understanding of IRI may be filled in the next years with new data derived from more sophisticated animal models and results of large randomized controlled trials.

References

1. Klein AS, Messersmith EE, Ratner LE, Kochik R, Baliga PK, Ojo AO. Organ donation and utilization in the United States, 1999-2008. Am J Transplant 2010;10:973-86.
2. Tonelli M, Wiebe N, Knoll G, et al. Systematic review: kidney transplantation compared with dialysis in clinically relevant outcomes. Am J Transplant 2011;11:2093-109.
3. Gjertson DW, Cecka JM. Living unrelated donor kidney transplantation. Kidney Int 2000;58:491-9.
4. Chkhotua AB, Klein T, Shabtai E, et al. Kidney transplantation from living-unrelated donors: comparison of outcome with living-related and cadaveric transplants under current immunosuppressive protocols. Urology 2003;62:1002-6.
5. Sapir-Pichhadze R, Young A, Joseph Kim S. Living donor age and kidney transplant outcomes: an assessment of risk across the age continuum. Transpl Int 2013;26:493-501.
6. Le Dinh H, Weekers L, Bonvoisin C, et al. Delayed graft function does not harm the future of donation-after-cardiac death in kidney transplantation. Transplant Proc 2012;44:2795-802.
7. Schuurs TA, Morariu AM, Ottens PJ, et al. Time-dependent changes in donor brain death related processes. Am J Transplant 2006;6:2903-11.
8. Nijboer WN, Moers C, Leuvenink HG, Ploeg RJ. How important is the duration of the brain death period for the outcome in kidney transplantation? Transpl Int 2011;24:14-20.
9. Nagaraja P, Roberts GW, Stephens M, et al. Influence of delayed graft function and acute rejection on outcomes after kidney transplantation from donors after cardiac death. Transplantation 2012;94:1218-23.
10. Wadei HM, Heckman MG, Rawal B, et al. Comparison of kidney function between donation after cardiac death and donation after brain death kidney transplantation. Transplantation 2013;96:274-81.
11. 2011. at http://www.eurotransplant.org/cms/mediaobject.php?file=year_20131.pdf.)
12. Pascual J, Zamora J, Pirsch JD. A systematic review of kidney transplantation from expanded criteria donors. Am J Kidney Dis 2008;52:553-86.
13. Martinez-Vaquera S, Navarro Cabello MD, Lopez-Andreu M, et al. Outcomes in renal transplantation with expanded-criteria donors. Transplant Proc 2013;45:3595-8.
14. Balaz P, Rokosny S, Wohlfahrtova M, et al. Identification of expanded-criteria donor kidney grafts at lower risk of delayed graft function. Transplantation 2013;96:633-8.
15. Domagala P, Kwiatkowski A, Perkowska-Ptasinska A, et al. Assessment of kidneys procured from expanded criteria donors before transplantation. Transplant Proc 2009;41:2966-9.
16. Johnson KJ, Weinberg JM. Postischemic renal injury due to oxygen radicals. Curr Opin Nephrol Hypertens 1993;2:625-35.
17. Kusch A, Hoff U, Bubalo G, et al. Novel signalling mechanisms and targets in renal ischaemia and reperfusion injury. Acta Physiol (Oxf) 2013;208:25-40.
18. Jang HR, Ko GJ, Wasowska BA, Rabb H. The interaction between ischemia-reperfusion and immune responses in the kidney. J Mol Med (Berl) 2009;87:859-64.
19. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004;364:1814-27.
20. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest 2011;121:4210-21.
21. Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 2008;40:3279-88.

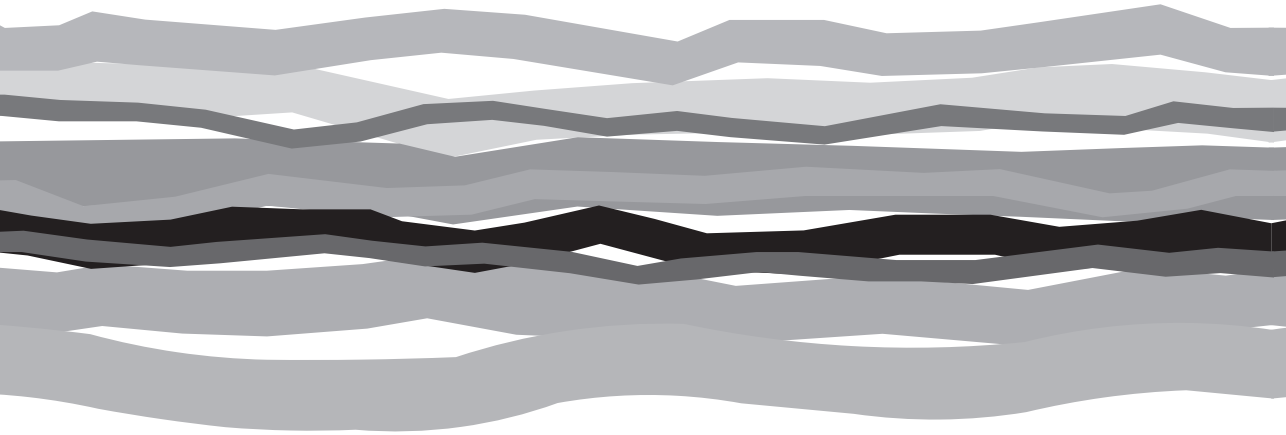
22. Ali S, Sheerin NS. Biomarkers of acute injury: predicting the long-term outcome after transplantation. Kidney Int 2013;84:1072-4.
23. Devarajan P, Mishra J, Supavekin S, Patterson LT, Steven Potter S. Gene expression in early ischemic renal injury: clues towards pathogenesis, biomarker discovery, and novel therapeutics. Mol Genet Metab 2003;80:365-76.
24. Fletcher JT, Nankivell BJ, Alexander SI. Chronic allograft nephropathy. Pediatr Nephrol 2009;24:1465-71.
25. van der Vliet JA, Warle MC. The need to reduce cold ischemia time in kidney transplantation. Curr Opin Organ Transplant 2013;18:174-8.
26. Thurman JM. Triggers of inflammation after renal ischemia/reperfusion. Clin Immunol 2007;123:7-13.
27. Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, et al. Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. Transplantation 2003;76:50-4.
28. Robertson LT, Mitchell JR. Benefits of short-term dietary restriction in mammals. Exp Gerontol 2013.
29. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. Biochem J 2013;449:1-10.
30. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010;9:40-53.
31. Verweij M, van de Ven M, Mitchell JR, et al. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation 2011;92:752-8.
32. Peng W, Robertson L, Gallinetti J, et al. Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. Sci Transl Med 2012;4:118ra11.
33. Mair W, Piper MD, Partridge L. Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS Biol 2005;3:e223.
34. Min KJ, Tatar M. Restriction of amino acids extends lifespan in Drosophila melanogaster. Mech Ageing Dev 2006;127:643-6.
35. Nygren J. The metabolic effects of fasting and surgery. Best Pract Res Clin Anaesthesiol 2006;20:429-38.
36. Stuart PC. The evidence base behind modern fasting guidelines. Best Pract Res Clin Anaesthesiol 2006;20:457-69.
37. Redman LM, Heilbronn LK, Martin CK, et al. Effect of calorie restriction with or without exercise on body composition and fat distribution. J Clin Endocrinol Metab 2007;92:865-72.
38. Rochon J, Bales CW, Ravussin E, et al. Design and conduct of the CALERIE study: comprehensive assessment of the long-term effects of reducing intake of energy. J Gerontol A Biol Sci Med Sci 2011;66:97-108.
39. van Ginhoven TM, de Bruin RW, Timmermans M, Mitchell JR, Hoeijmakers JH, Ijzermans JN. Preoperative dietary restriction is feasible in live-kidney donors. Clin Transplant 2011;25:486-94.
40. Hoeger S, Lueg G, Tsagogiorgas C, et al. UW is superior compared with HTK after prolonged preservation of renal grafts. J Surg Res 2011;170:e149-57.
41. Straatsburg IH, Abrahamse SL, Song SW, Hartman RJ, Van Gulik TM. Evaluation of rat liver apoptotic and necrotic cell death after cold storage using UW, HTK, and Celsior. Transplantation 2002;74:458-64.

42. Stubenitsky BM, Ametani M, Danielewicz R, Southard JH, Belzer FO. Regeneration of ATP in kidney slices after warm ischemia and hypothermic preservation. Transpl Int 1995;8:293-7.
43. Chatauret N, Thuillier R, Hauet T. Preservation strategies to reduce ischemic injury in kidney transplantation: pharmacological and genetic approaches. Curr Opin Organ Transplant 2011;16:180-7.
44. Moers C, Smits JM, Maathuis MH, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 2009;360:7-19.
45. Burgos Revilla FJ, Hevia V, Diez V, et al. Machine perfusion: initial results in an expanded criteria donor kidney transplant program. Transplant Proc 2015;47:19-22.
46. Jochmans I, O'Callaghan JM, Pirenne J, Ploeg RJ. Hypothermic machine perfusion of kidneys retrieved from standard and high-risk donors. Transpl Int 2015.
47. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. Am J Transplant 2013;13:1246-52.
48. Deng R, Gu G, Wang D, et al. Machine perfusion versus cold storage of kidneys derived from donation after cardiac death: a meta-analysis. PLoS One 2013;8:e56368.
49. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? Transpl Int 2015;28:657-64.
50. Kierulf-Lassen C, Nieuwenhuijs-Moeke GJ, Krogstrup NV, Oltean M, Jespersen B, Dor FJ. Molecular Mechanisms of Renal Ischemic Conditioning Strategies. Eur Surg Res 2015;55:151-83.
51. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124-36.
52. Candilio L, Malik A, Hausenloy DJ. Protection of organs other than the heart by remote ischemic conditioning. J Cardiovasc Med (Hagerstown) 2013;14:193-205.
53. Wever KE, Menting TP, Rovers M, et al. Ischemic preconditioning in the animal kidney, a systematic review and meta-analysis. PLoS One 2012;7:e32296.
54. Riksen NP, Hausenloy DJ, Yellon DM. Erythropoietin: ready for prime-time cardioprotection. Trends Pharmacol Sci 2008;29:258-67.
55. Hausenloy DJ, Mwamure PK, Venugopal V, et al. Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. Lancet 2007;370:575-9.
56. Jiang H, Chen R, Xue S, Zhu H, Sun X, Sun X. Protective effects of three remote ischemic conditioning procedures against renal ischemic/reperfusion injury in rat kidneys: a comparative study. Ir J Med Sci 2014.
57. Bedir S, Ma Y, Antonelli J, Cadeddu JA, Gahan JC. Ineffectiveness of Remote Ischemic Renal Preconditioning in a Porcine Solitary-Kidney Model. J Endourol 2015.
58. Wu J, Feng X, Huang H, et al. Remote ischemic conditioning enhanced the early recovery of renal function in recipients after kidney transplantation: a randomized controlled trial. J Surg Res 2014;188:303-8.
59. Krogstrup NV, Oltean M, Bibby BM, et al. Remote ischaemic conditioning on recipients of deceased renal transplants, effect on immediate and extended kidney graft function: a multicentre, randomised controlled trial protocol (CONTEXT). BMJ Open 2015;5:e007941.
60. Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579-88.
61. Mykytenko J, Kerendi F, Reeves JG, et al. Long-term inhibition of myocardial infarction by postconditioning during reperfusion. Basic Res Cardiol 2007;102:90-100.

62. Hausenloy DJ, Yellon DM. The therapeutic potential of ischemic conditioning: an update. Nat Rev Cardiol 2011;8:619-29.
63. van den Akker EK, Manintveld OC, Hesselink DA, de Bruin RW, Ijzermans JN, Dor FJ. Protection against renal ischemia-reperfusion injury by ischemic postconditioning. Transplantation 2013;95:1299-305.
64. Eldaif SM, Deneve JA, Wang NP, et al. Attenuation of renal ischemia-reperfusion injury by postconditioning involves adenosine receptor and protein kinase C activation. Transpl Int 2010;23:217-26.
65. Jiang B, Liu X, Chen H, et al. Ischemic postconditioning attenuates renal ischemic/reperfusion injury in mongrel dogs. Urology 2010;76:1519 e1-7.
66. Miklos Z, Kurthy M, Degrell P, et al. Ischaemic postconditioning reduces serum and tubular TNF-alpha expression in ischaemic-reperfused kidney in healthy rats. Clin Hemorheol Microcirc 2012;50:167-78.
67. Weng X, Shen H, Kuang Y, et al. Ischemic postconditioning inhibits the renal fibrosis induced by ischemia-reperfusion injury in rats. Urology 2012;80:484 e1-7.
68. Kadkhodaee M, Seifi B, Najafi A, Sedaghat Z. First report of the protective effects of remote per- and postconditioning on ischemia/reperfusion-induced renal injury. Transplantation 2011;92:e55.
69. van den Akker EK, Hesselink DA, Manintveld OC, et al. Ischemic postconditioning in human DCD kidney transplantation is feasible and appears safe. Transpl Int 2014;27:226-34.
70. Masoud MS, Anwar SS, Afzal MZ, Mehmood A, Khan SN, Riazuddin S. Pre-conditioned mesenchymal stem cells ameliorate renal ischemic injury in rats by augmented survival and engraftment. J Transl Med 2012;10:243.
71. Chen YT, Sun CK, Lin YC, et al. Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. J Transl Med 2011;9:51.
72. Zhuo W, Liao L, Xu T, Wu W, Yang S, Tan J. Mesenchymal stem cells ameliorate ischemia-reperfusion-induced renal dysfunction by improving the antioxidant/oxidant balance in the ischemic kidney. Urol Int 2011;86:191-6.
73. Zhuo W, Liao L, Fu Y, et al. Efficiency of endovenous versus arterial administration of mesenchymal stem cells for ischemia-reperfusion-induced renal dysfunction in rats. Transplant Proc 2013;45:503-10.
74. Cao H, Qian H, Xu W, et al. Mesenchymal stem cells derived from human umbilical cord ameliorate ischemia/reperfusion-induced acute renal failure in rats. Biotechnol Lett 2010;32:725-32.
75. Semedo P, Palasio CG, Oliveira CD, et al. Early modulation of inflammation by mesenchymal stem cell after acute kidney injury. Int Immunopharmacol 2009;9:677-82.
76. Behr L, Hekmati M, Lucchini A, et al. Evaluation of the effect of autologous mesenchymal stem cell injection in a large-animal model of bilateral kidney ischaemia reperfusion injury. Cell Prolif 2009;42:284-97.
77. Behr L, Hekmati M, Fromont G, et al. Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 2007;107:p65-76.
78. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. Stem Cells Dev 2013;22:2825-35.
79. Burst VR, Gillis M, Putsch F, et al. Poor cell survival limits the beneficial impact of mesenchymal stem cell transplantation on acute kidney injury. Nephron Exp Nephrol 2010;114:e107-16.
80. Luo J, Zhao X, Tan Z, Su Z, Meng F, Zhang M. Mesenchymal-like progenitors derived from human embryonic stem cells promote recovery from acute kidney injury via paracrine actions. Cytotherapy 2013;15:649-62.

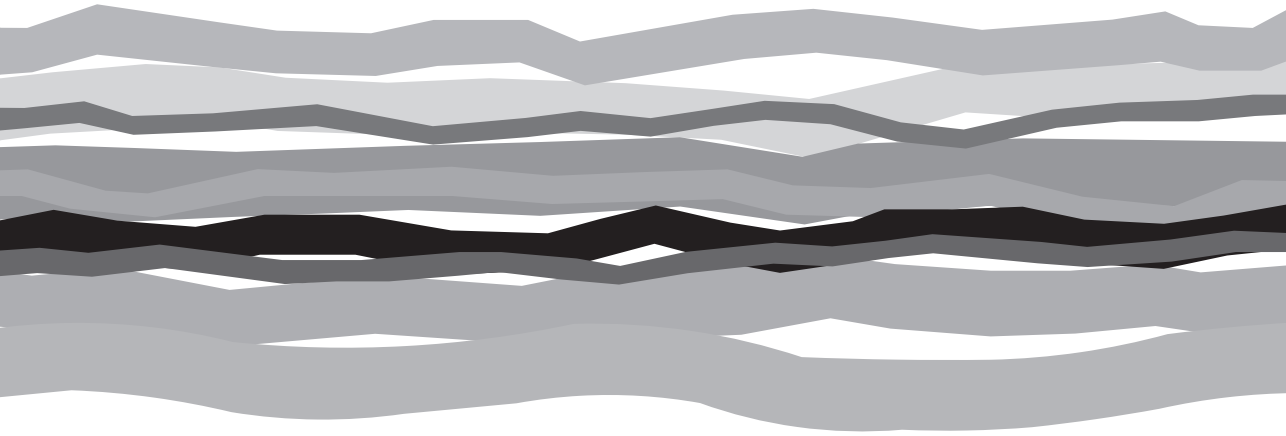
81. Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428:664-8.
82. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428:668-73.
83. Perico N, Casiraghi F, Introna M, et al. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. Clin J Am Soc Nephrol 2011;6:412-22.
84. Reinders ME, de Fijter JW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Transl Med 2013;2:107-11.
85. Peng Y, Ke M, Xu L, et al. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. Transplantation 2013;95:161-8.
86. Luk F, de Witte SF, Bramer WM, Baan CC, Hoogduijn MJ. Efficacy of immunotherapy with mesenchymal stem cells in man: a systematic review. Expert Rev Clin Immunol 2015;11:617-36.
87. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood 2011;117:1061-70.
88. Kinsey GR, Sharma R, Okusa MD. Regulatory T cells in AKI. J Am Soc Nephrol 2013;24:1720-6.
89. Kinsey GR, Sharma R, Huang L, et al. Regulatory T cells suppress innate immunity in kidney ischemia-reperfusion injury. J Am Soc Nephrol 2009;20:1744-53.
90. Dijke IE, Hoeppli RE, Ellis T, et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. Am J Transplant 2015.
91. Bajwa A, Kinsey GR, Okusa MD. Immune mechanisms and novel pharmacological therapies of acute kidney injury. Curr Drug Targets 2009;10:1196-204.
92. Attallah N, Yassine L, Musial J, Yee J, Fisher K. The potential role of statins in contrast nephropathy. Clin Nephrol 2004;62:273-8.
93. Khanal S, Attallah N, Smith DE, et al. Statin therapy reduces contrast-induced nephropathy: an analysis of contemporary percutaneous interventions. Am J Med 2005;118:843-9.
94. Jo SH, Koo BK, Park JS, et al. Prevention of radiocontrast medium-induced nephropathy using short-term high-dose simvastatin in patients with renal insufficiency undergoing coronary angiography (PROMISS) trial--a randomized controlled study. Am Heart J 2008;155:499 e1-8.
95. Philips B, MacPhee I. Do statins prevent acute kidney injury? Expert Opin Drug Saf 2015;14:1547-61.
96. Ishii Y, Sawada T, Murakami T, et al. Renoprotective effect of erythropoietin against ischaemia-reperfusion injury in a non-human primate model. Nephrol Dial Transplant 2011;26:1157-62.
97. Zhang J, Zou YR, Zhong X, et al. Erythropoietin pretreatment ameliorates renal ischaemia-reperfusion injury by activating PI3K/Akt signalling. Nephrology (Carlton) 2015;20:266-72.
98. Song YR, Lee T, You SJ, et al. Prevention of acute kidney injury by erythropoietin in patients undergoing coronary artery bypass grafting: a pilot study. Am J Nephrol 2009;30:253-60.
99. Xin H, Ge YZ, Wu R, et al. Effect of high-dose erythropoietin on graft function after kidney transplantation: a meta-analysis of randomized controlled trials. Biomed Pharmacother 2015;69:29-33.
100. Vlachopanos G, Kassimatis TI, Agrafiotis A. Perioperative administration of high-dose recombinant human erythropoietin for delayed graft function prevention in kidney transplantation: a meta-analysis. Transpl Int 2015;28:330-40.
101. Brines M, Patel NS, Villa P, et al. Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. Proc Natl Acad Sci U S A 2008;105:10925-30.

102. Yang C, Xu Z, Zhao Z, et al. A novel proteolysis-resistant cyclic helix B peptide ameliorates kidney ischemia reperfusion injury. Biochim Biophys Acta 2014;1842:2306-17.
103. Yang C. Cyclic helix B peptide in preservation solution and autologous blood perfusate ameliorates ichemia-reperfusion injury in isolated porcine kidneys. Transplantation Direct 2015.
104. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97.
105. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. Clin Chem 2010;56:1733-41.
106. Sui W, Dai Y, Huang Y, Lan H, Yan Q, Huang H. Microarray analysis of MicroRNA expression in acute rejection after renal transplantation. Transpl Immunol 2008;19:81-5.
107. Anglicheau D, Sharma VK, Ding R, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci U S A 2009;106:5330-5.
108. Godwin JG, Ge X, Stephan K, Jurisch A, Tullius SG, Iacomini J. Identification of a microRNA signature of renal ischemia reperfusion injury. Proc Natl Acad Sci U S A 2010;107:14339-44.
109. van den Akker EK, Dor FJ, JN IJ, de Bruin RW. MicroRNAs in Kidney Transplantation: Living up to Their Expectations? J Transplant 2015;2015:354826.
110. Rowinski W, Paczek L. Transplantation ethics: are we approaching the crossroads? Transplant Proc 2012;44:2171-2.
111. Sachs DH. Tolerance: of mice and men. J Clin Invest 2003;111:1819-21.
112. Sachdeva M, Sunday S, Israel E, et al. Obesity as a barrier to living kidney donation: a center-based analysis. Clin Transplant 2013.
113. Hourmant M, Lerat L, Karam G. Donation from old living donors: how safe is it? Nephrol Dial Transplant 2013;28:2010-4.
114. Chapman JR, Nankivell BJ. Nephrotoxicity of ciclosporin A: short-term gain, long-term pain? Nephrol Dial Transplant 2006;21:2060-3.
115. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med 2003;349:2326-33.
116. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Chapman JR, Allen RD. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. Transplantation 2004;78:557-65.
117. Budde K, Becker T, Arns W, et al. Everolimus-based, calcineurin-inhibitor-free regimen in recipients of de-novo kidney transplants: an open-label, randomised, controlled trial. Lancet 2011;377:837-47.
118. Ponticelli C. Herpes viruses and tumours in kidney transplant recipients. The role of immunosuppression. Nephrol Dial Transplant 2011;26:1769-75.
119. Carroll RP, Ramsay HM, Fryer AA, Hawley CM, Nicol DL, Harden PN. Incidence and prediction of nonmelanoma skin cancer post-renal transplantation: a prospective study in Queensland, Australia. Am J Kidney Dis 2003;41:676-83.
120. ter Riet G, Korevaar DA, Leenaars M, et al. Publication bias in laboratory animal research: a survey on magnitude, drivers, consequences and potential solutions. PLoS One 2012;7:e43404.



Chapter 3

A comparison of inflammatory, cytoprotective and injury gene expression profiles in kidneys from brain death and cardiac death donors



Tanja C. Saat, Denis Susa, Henk P. Roest, Niels F.M. Kok, Sandra van den Engel, Jan N.M. IJzermans, Ron W.F. de Bruin

Transplantation

Abstract

The superior long-term survival of kidneys from Living Donors (LD) compared to kidneys from Donation after Brain Death (DBD) and Donation after Circulatory Death (DCD) is now well established. However, comparative studies on transcriptional changes that occur at organ retrieval, and during and after cold ischemia (CI) are sparse. Using a rat model, we used qRT-PCR to examine expression levels of inflammatory, cytoprotective and injury genes at different time points after organ retrieval. Cleaved caspase-3, was used to evaluate early apoptosis in DCD- and DBD kidneys. Immediately after retrieval we found massive up-regulation of pro-inflammatory genes IL-1β, IL-6, TNF-α, MCP-1, P-selectin and E-selectin in DBD- compared to LD- and DCD kidneys. A significant increase in the expression of injury markers Kim-1, and p21, and the cytoprotective gene HO-1 accompanied this. Bax was up-regulated in DCD kidneys, and Bcl-2 was down-regulated in DBD kidneys. After 2 hours of CI in the LD group and 18 hours in the DBD and DCD groups, gene expression levels were similar to those found after retrieval. During 18 hours of cold storage expression levels of these genes did not change. In DCD- and DBD kidneys, early apoptosis increased after CI. The gene expression profile in DBD kidneys represents an inflammatory and injury response to brain death. In contrast, DCD kidneys show only mild up-regulation of inflammatory and injury genes. These results may imply why delayed graft function in DCD kidneys does not have the deleterious effect it has in DBD kidneys.

Introduction

The superior long-term survival of kidney transplants from Living Donors (LD) compared to Donation after Brain Death (DBD) or Donation after Circulatory Death (DCD) donors are now well established[1-3]. LD are healthy during organ procurement, and retrieved kidneys are exposed to minimal warm and cold ischemia (CI) times. DBD donors suffer from hemodynamic instability, hormone dysregulation and immunologic reactivity, which contribute to organ damage and inferior post-transplant function and graft survival compared to LD[4-8]. Organs from DCD donors do not develop the physiological changes of DBD donors, but are exposed to prolonged warm ischemia times (WIT), which increase the incidence of delayed graft function (DGF). In addition, organs from both post-mortem groups are exposed to prolonged CI compared to LD[9,10]. Although there is no difference in the incidence or severity of acute rejection between DBD- and DCD kidneys, DCD donors have a 73% chance of DGF compared to 27% in DBD kidneys[11]. However, the long-term graft survival of renal transplants of DCD- is similar to those from DBD donors[11-15]. Improved technology and an increasing gap between supply and demand for donor organs have prompted an increased use of organs from both LD and DCD donors[4,16].

The pathophysiology that leads to organ damage following brain death is relatively well established[5,17-19], and strategies to protect the organs of a DBD donor are emerging[20-23]. In contrast, experimental studies on the changes that occur in organs from DCD donors are sparse[24]. A drawback in clinical studies that compare peri-operative gene expression patterns during transplantation is that they are confined to a relatively small time-span, and a limited number of time-points. Usually "t=0 biopsies" are obtained at the end of the cold storage period, followed by a post-reperfusion biopsy 30-60 minutes after reperfusion of the graft[19]. These time-points are not able to take into account the major pathophysiological perturbances that occur during the periods of brain death, cardiac arrest, organ retrieval and the subsequent cold storage period. Experimental studies have tried to overcome these limitations by studying gene expression at explantation, but have mainly focused on DBD donors[25,26].

The aim of this study therefore was to compare expression levels of genes indicative of inflammation (IL-6, IL-1α, TNF-α, MCP-1, E-selectin, P-selectin, TLR4, HMGB1), cytoprotection (HO-1) and injury (HIF-1α, VEGF, Bax, Bcl-2, p21, Kim-1) at the time of kidney retrieval, after clinically relevant CI times and over a time-course during cold storage.

Materials and Methods

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe. Male Brown Norway rats of 12-14 weeks old, weighing 250–300g, were purchased from Harlan-CPB (The Netherlands). Rats were randomly assigned to a LD, DBD or DCD group (n=7/group). The LD group served as control for both post-mortem groups.

After kidney retrieval, samples were stored in University of Wisconsin (UW) solution; time-point 0 samples were directly snap frozen in nitrogen. At 2, 4, 6, 12 and 18 hours the kidneys were collected and snap frozen until further use. Gene expression levels after retrieval were measured in time-point 0 samples. To measure expression levels after clinically relevant CI; 2 hours of CI was used for LD and 18 hours of CI for the post-mortem donors. mRNA expression levels were examined by qRT-PCR. For immunohistochemistry DCD- and DBD kidneys were used directly after retrieval and after 18 hours of CI.

Experimental models

Animals were anesthetized with isoflurane. After intubation, anaesthesia was maintained using a mixture of N_2O/O_2/2% isoflurane. Animals were pressure control ventilated on a Siemens Servo 900C ventilator with 14 cm H_2O PIP, 4 cm H_2O PEEP with a frequency of 40 breaths per minute.

In the DBD group a frontolateral trepanation was made and a balloon catheter (Fogarty Arterial Embolectomy Catheter: 5F, BaxterHealthcare Co.) was introduced in the extradural space and slowly inflated, causing a gradually increasing intra-cranial pressure[27]. Herniation of the brainstem and DBD was confirmed by dilated and fixed pupils, the absence of corneal reflexes, and an apnoea test. Brain dead animals received ventilation but no further anaesthesia. Intra-arterial pressure was continuously monitored via a PE50 catheter placed in the carotid artery. Only rats with stable mean arterial pressure (MAP > 80 mm Hg) during the 6 hours of brain death were included in the experiment. After 6 hours both kidneys were removed, flushed with PBS and stored in UW solution at 4°C.

In the DCD group the animals were catheterised using a PE50 catheter placed in the carotid artery, and both kidneys were exposed via a midline incision that was then temporarily closed with a few sutures until retrieval of the kidneys. Cardiac arrest was induced by isoflurane overdose and lasted 20 minutes, starting when the blood pressure had dropped to 5-7 mm Hg, the time commonly needed before starting with cold storage of DCD donor kidneys. The DCD donors are type III, controlled; waiting for cardiac

arrest, of the Maastricht criteria[28]. In the LD group, nephrectomy was performed after one hour of mechanical ventilation.

mRNA expression analysis

Kidney sections were homogenized in 1.0 ml Trizol (Invitrogen). Total RNA was isolated according to the manufacturer's guidelines, precipitated, and subsequently dissolved in DEPC-treated water. RNA concentration was determined using a Nanodrop ND-1000 UV-VIS spectrophotometer. For cDNA synthesis M-MLV Reverse Transcriptase (Invitrogen) was used according to the manufacturer's protocol with 250ng of random hexamers (Promega) and RNAseOUT (Invitrogen). All temperature-dependent steps were performed using a Biometra T-gradient cycler (Biometra Gmbh). cDNA samples were stored at -20°C. Quantitative real-time PCR was performed using a BioRad Icycler with SYBR Green incorporation. Each sample was tested in duplo, two times. ΔCt values of genes of interest were calculated as described by Pfaffl et al.[29] using β-actin as a reference gene. ΔCt values were normalized to the average ΔCt of the LD. LD kidneys 2 hours after CI were used comparison for the post-mortem kidneys after 18 hours of CI, and the respective time-points in the time-course. The fold increase was calculated using the Pfaffl equation: $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM.

Primers

Inflammation (IL-6, IL-1α, TNF-α, MCP-1, E-selectin, P-selectin, TLR4, HMGB1), cytoprotection (HO-1) and injury (HIF-1α, VEGF, Bax, Bcl-2, p21, Kim-1)

Primers indicative for inflammation are TLR4, HMGB1, IL-1β, IL-6, TNF-α, MCP-1, P-selectin and E-selectin. Primers indicative for cytoprotection are HO-1, VEGF and HIF-1α. Kim-1, p21, Bax and Bcl-2 were used as markers indicative for kidney injury. Used primers are shown in **Table 1**.

Immunohistochemistry

Frozen sections, 5µm-thick, were stained with a polyclonal antibody against CC3 (Asp175; Cell Signaling), an early apoptotic marker. CC3 was diluted 1:300 and visualized with a horseradish peroxidase-conjugated secondary antibody (dilution 1:500, goat-anti-rabbit IgG/HRP, DAKO, Denmark). Slides were scored at a magnification of 200x by 2 independent observers blinded to the treatment. Results are expressed as mean ± SEM.

Statistical Analysis

Data were analysed using a Kruskal Wallis ANOVA, followed by a Mann-Whitney–U test. Analysis was performed using SPSSv20.0. A p value ≤0.05 was considered statistically significant.

Table 1. Primers used for qRT-PCR.

Housekeeping Gene	Forward Primer	Reverse Primer
β-actin	'5-gacccagatcatgtttgagacc-3'	'5-gatgggcacagtgtgggtgac-3'
Inflammatory Genes	**Forward Primer**	**Reverse Primer**
IL-1β	'5-gaggctgacagaccccaaaaga-3'	'5-tccacagccacaatgagtgaca-3'
IL-6	'5-gtctcgagcccaccaggaac-3'	'5-agggaaggcagtggctgtca-3'
TNF-α	'5-gaccctcacactcagatcatcttct-3'	'5-tgctacgacgtgggctacg-3'
MCP-1	'5-gccatcagcccacaggtgtt-3'	'5-gggacactggctgcttgtga-3'
P-selectin	'5-aagatggtcagcgcctccac-3'	'5-atcgaaccgatgggacagga-3'
E-selectin	'5-cccacatgtgcaggggtaca-3'	'5-tggcccactgcaactcatgt-3'
TLR4	'5-tccgctggttgcagaaaatg-3'	'5-tcccagccagatgcaagaga-3'
HMGB1	'5-ccgccaagctgaaggagaag-3'	'5-ccctttttcgctgcatcagg-3'
Cytoprotective Genes	**Forward Primer**	**Reverse Primer**
HO-1	'5-tttcagaagggtcaggtgtcca-3'	'5-agtagagcggggcatagactgg-3'
p21	'5-ccacagcgatatcgagacactca-3'	'5-acagacgacggcatactttgctc-3'
Injury Genes	**Forward Primer**	**Reverse Primer**
HIF-1α	'5-agtcagcaacgtggaaggtgc-3'	'5-acaaatcagcaccaagcacgtc-3'
VEGF	'5-tgcactggaccctggctttac-3'	'5-ttctgctccccttctgtcgtg-3'
Bax	'5-tgctgatggcaacttcaactgg-3'	'5-tggttctgatcagctcgggca-3'
Bcl-2	'5-acatcgccctgtggatgactg-3'	'5-gcatgctggggccatatagttc-3'
Kim-1	'5-ccacagcgatatcgagacactca-3'	'5-acagacgacggcatactttgctc-3'

Results

Expression of pro-inflammatory, cytoprotective and injury genes after retrieval

Directly after retrieval, pro-inflammatory genes showed a significant increase in DBD- compared to LD kidneys (**Figure 1**). Intrarenal IL-1β mRNA levels were increased in DBD- (13.7-fold, p=0.032) versus LD kidneys. IL-6 expression was massively increased in the DBD- compared to the LD kidneys (417-fold, p=0.003). TNF-α and MCP-1 (not shown) were increased (4.7-fold, p=0.004) and (81-fold, p=0.015), respectively. In DCD kidneys MCP-1, IL-1β and IL-6 were not significantly different compared to LD, while TNF-α was slightly but significantly lower (p=0.003). Expression of the adhesion molecules P-selectin and E-selectin was highly elevated in DBD-, but not in DCD kidneys (not shown). Cytoprotective gene HO-1 was highly up-regulated in DBD- compared to LD kidneys (36.7-fold, p=0.004), whereas no difference was found between the DCD and LD group. Neither HIF-1α, nor VEGF expression was significantly influenced by DBD or DCD compared to LD. The anti-apoptotic gene Bax (not shown) and pro-apoptotic Bcl-2 (not shown) showed no significant differences between the groups. Cell-cyle inhibitor p21 was significantly elevated in both DBD- (5.0-fold, p=0.032) and DCD kidneys (1.6-fold,

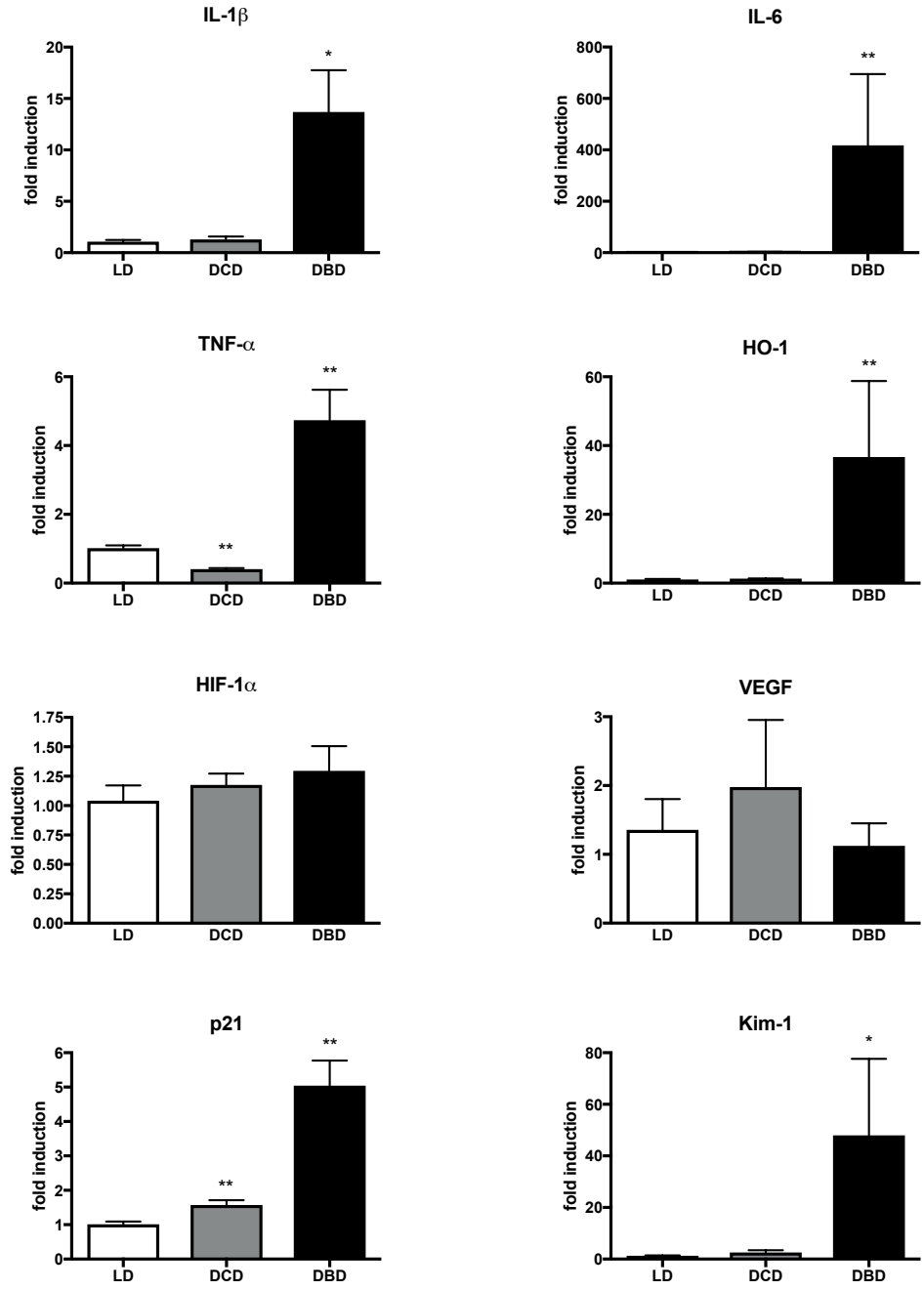


Figure 1. mRNA expression levels of genes indicative of inflammation, cytoprotection, and kidney injury were determined by qRT-PCR directly after retrieval of the kidneys in living donors (LD), donation after circulatory death (DCD) and donation after brain death (DBD) donors. To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at time point 0. Subsequently the fold increase was calculated using 2ΔΔCt. Results are expressed as mean ± SEM of seven animals per group. *p<0.05, **p<0.01 compared to LD.

p=0.007). These changes were accompanied by a significant up-regulation of kidney injury marker Kim-1 in DBD (48-fold, p=0.021).

Gene expression levels after clinically relevant cold ischemia times

Next, we investigated the expression levels after CI relevant to human kidney transplantation. LD kidneys were analysed after 2 hours of CI, and the DBD and DCD groups after 18 hours of CI[30,31].

Compared to LD, IL-1β, IL-6, TNF-α and MCP-1 were increased in DBD kidneys (43-fold, p=0.003; 997-fold, p=0.003; 9.7-fold, p=0.003 and 115-fold, p=0.004). IL-1β and IL-6 were increased in DCD kidneys but to a lesser extend (2.3-fold, p=0.004; 10-fold, p=0.003). TNF-α and MCP-1 showed no significant difference in expression between DCD and LD kidneys. P-selectin was significantly increased in DBD- (129-fold, p=0.003) and DCD kidneys (2.9-fold, p=0.008). E-selectin was increased in DBD kidneys (64-fold, p=0.003). TLR4 expression was elevated in DBD- (4.4-fold, p=0.037), but not in DCD kidneys. HMGB1 was not significantly different.

After DBD, HO-1 was up-regulated (21-fold, p=0.001), while in the DCD donors HO-1 was slightly but significantly lower compared to the LD (p=0.014). Neither HIF-1α, nor VEGF expression was significantly influenced by DBD or DCD. Bax was expressed higher in the DCD compared to the LD kidney (1.3-fold, p=0.016). Bcl-2 was lower in DBD- compared to LD kidneys (0.7-fold, p=0.032). Expression of p21 was higher in both DBD- and DCD donors, (9.1-fold, p=0.003) and (2.5-fold, p=0.003), as compared to LD. In DBD donors Kim-1 was increased (319-fold, p=0.003), whereas in DCD donors it was slightly increased (5.3-fold, p=0.010).

Gene expression profiles during an 18 hour cold storage period

To address whether changes in gene-expression would occur during cold storage of the kidneys, gene expression of HO-1, HIF-1α, Bax, Bcl-2, TLR4, HMGB1 and VEGF (not shown) were examined after 0, 2, 4, 6, 12 and 18 hours of cold storage (**Figure 2**). Gene expression levels did not significantly change after retrieval and during cold storage of the kidney grafts. These data show that biological processes in the donor are the main determinants of gene expression in the graft prior to transplantation.

Apoptosis during cold storage

DCD- and DBD kidneys collected directly after retrieval and after 18 hours of CI were stained for cleaved caspase-3 (CC3) (**Figure 3**). In DCD kidneys, the average number of apoptotic cells increased after 18 hours of Ci compared with kidneys directly after retrieval (p<0.01). DBD kidneys showed a similar trend.

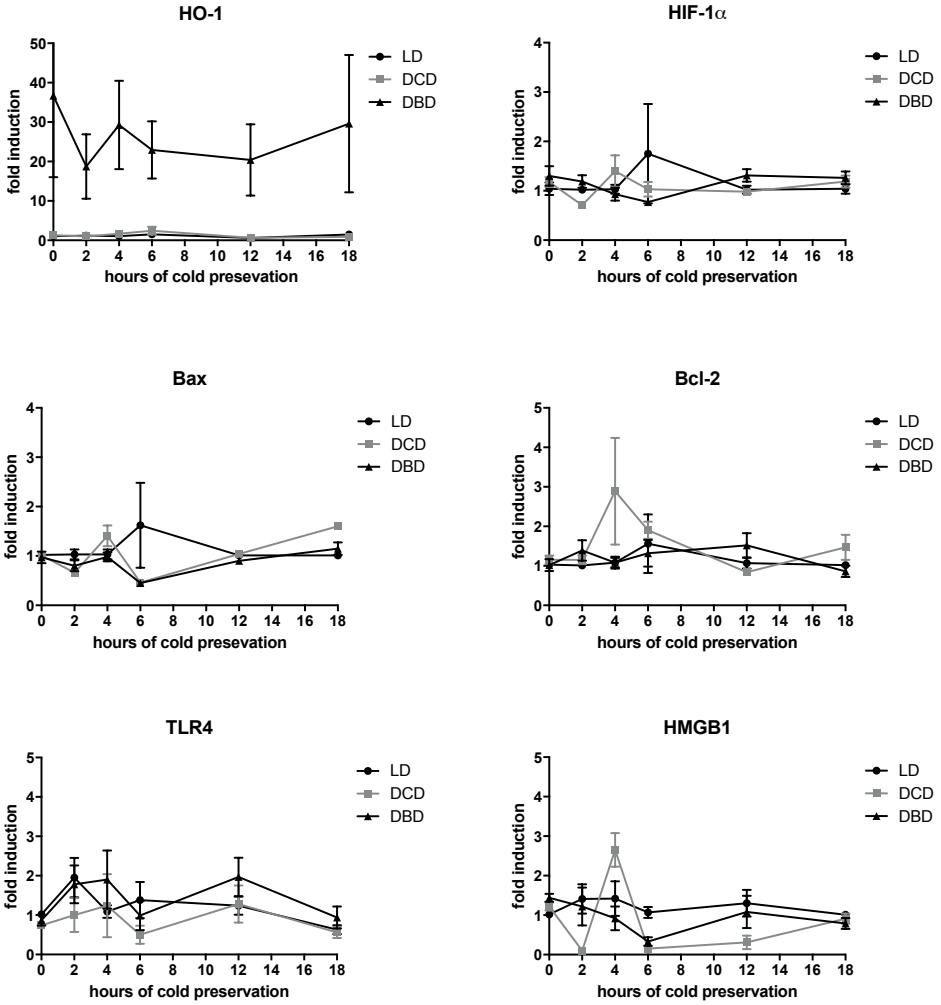


Figure 2. Gene expression profiles in kidneys remain stable during cold preservation. mRNA expression levels of HO-1, HIF-1α, Bax, Bcl-2, TLR-4 and HMGB1 were determined directly after retrieval of kidneys from the donor, and at 2, 4, 6, 12, and 18 hours of cold preservation in kidneys of living donors (LD), donation after circulatory death (DCD,) and brain death (DBD) donors. To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at the respective time points. Subsequently the fold increase was calculated using $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM of seven animals per group.

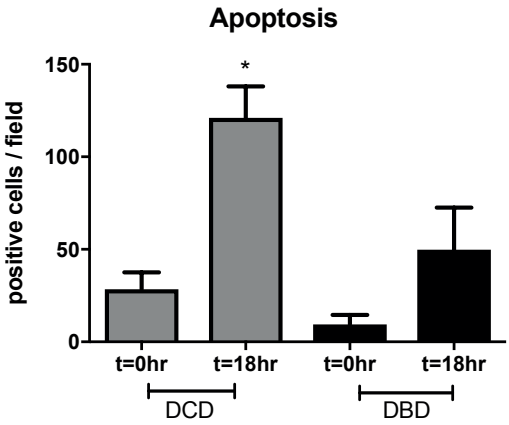


Figure 3. Early apoptosis was analysed using a cleaved caspase-3 staining in DCD- and DBD kidneys after retrieval and after 18 hours of cold ischemia. Directly after retrieval DCD kidneys showed less apoptotic cells compared with DCD kidneys after 18 hours of cold preservation. DBD kidneys show a similar trend. *p<0.01 compared with time-point 0.

Discussion

To better understand the relative contributions of brain death, prolonged WIT and CI in DBD- and DCD donors, we studied expression profiles of genes representative of inflammation, cytoprotection, and injury. Directly after kidney retrieval, pro-inflammatory genes showed a massive up-regulation in DBD- compared to LD- and DCD kidneys. A significant increase in the expression of Kim-1 and p21, and cytoprotective gene HO-1 accompanied this. After clinically relevant CI times, the gene expression profiles remained stable. The high levels of pro-inflammatory and injury genes in DBD donors are in contrast with the clinically high incidence of DGF in DCD- compared to DBD donors. Induction of pro-inflammatory and injury genes alone might not be sufficient to induce DGF and influence short-term graft outcome. Other factors could play an import role in the incidence of DGF and post-transplant outcome[32], since DCD kidneys showed cellular changes during CI. In the clinical setting the average WIT in DCD donors is approximately 20 minutes[33]. Although the prolonged WIT in organs from DCD donors is considered the main reason for the increased incidence of DGF in these grafts, the deterioration in organ quality induced by 20 minutes of WIT in this study is not reflected by changes in gene expression. During cold preservation, hypothermia and the lack of oxygen preclude the transcription of DNA into mRNA. The use of preservation fluids preserves cellular integrity and may prevent breakdown of RNA, and this may explain the stable mRNA levels during cold preservation[34-37].

The most striking finding in our study is the difference between DBD- and DCD donors in renal transcription of inflammatory markers such as IL-1β, IL-6, MCP-1, and E-selectin.

High levels of inflammatory cytokines in the donor are associated with decreased graft survival[26,38,39]. The high levels of IL-6 in serum of DBD donors are thought to be due to production in the injured brain and subsequent leakage through the disrupted blood-brain barrier[17,40]. We show that it is likely that the kidney itself significantly contributes to systemic IL-6 levels, and systemic inflammation observed during brain death. This inflammation resulted in kidney injury, as shown by the up-regulation of Kim-1. In DCD kidneys, expression of IL-1β, IL-6 and P-selectin was significantly up-regulated compared to LD but to a much lesser extent than in DBD donors.

TLR4 expression was significantly up-regulated in DBD donors. The important role of TLR4 in mediating renal ischemia reperfusion injury has been shown both in animal models and after human transplantation[41,42]. HMGB1 has been identified as ligand for TLR4 present on tubular epithelial cells, and is involved in the cellular signalling through TLR4, which leads to production of pro-inflammatory cytokines[41]. HMGB1 secretion is stimulated by IL-1β, and by stressed cells or cells undergoing necrosis[43,44]. Up-regulation of both TLR4 and HMGB1 have been observed following reperfusion, and targeting both may diminish ischemic injury[42]. Although we found up-regulation of many cytokines that can be induced by TLR4 activation, such as TNF-α and MCP-1, we found low levels of HMGB1, which might suggest that it is not involved in the systemic inflammatory response observed during DBD in our model, or that low levels of HMGB1 are able to activate TLR4. In addition, TLR4 can also be activated by other alarmins[43].

Compared to the severe inflammatory response induced during 6 hours of brain death, 20 minutes of WIT in our DCD model induced only mild changes, which are almost indistinguishable from the transcriptional changes found after LD. Since the duration of the asystolic period in DCD donors determines the severity of the damage to the kidney[24], it is likely that 20 minutes of asystole used in our study is not sufficient to induce massive up-regulation of pro-inflammatory gene expression. In addition, human DCD donors may suffer from other injuries, which influence the inflammatory response. Because of the higher incidence of DGF in DCD donors[9,10,45], it has even been suggested that DGF in DCD kidneys predisposes for favourable graft outcome[46]. The absence of overt inflammatory changes and significantly lower Kim-1 expression in our study may explain why the impact of DGF is reduced in DCD kidneys.

We found a significant up-regulation of HO-1 in DBD donors at the time of retrieval, which remained stable during 18 hours of CI. Several studies describe that over-expression of HO-1 protects kidney transplants against the deleterious consequences of ischemic injury[47-49]. In LD elevated HO-1 expression in the transplant had a protective effect on renal ischemia reperfusion injury[25,50]. In contrast, the increased levels of HO-1 in DBD kidneys did not prevent renal damage. This suggests that DBD causes renal damage to such an extent that HO-1 expression is insufficient, and may be viewed as a marker of injury[25].

Both HIF-1α, and VEGF itself were not significantly up-regulated in DBD donors. This implies that during 6 hours of brain dead, the inflammatory response coincides with up-regulation of HO-1, not VEGF. Interestingly, p21 was also strongly induced in both post-mortem donors. p21 binds stoichiometrically to PCNA and inhibits its action in DNA replication, is a key regulator of proliferation following renal ischemic injury[51]. We previously showed that there is a critical, temporal balance between the expression levels of p21 and proliferation in the regenerating tubular compartment following ischemia[52]. The up-regulation of p21, like HO-1, in our model may be envisioned as indicator of damage, and the lower induction in DCD kidneys may give an advantage following transplantation, when proliferation of the damaged tubular compartment is warranted[51,52].

The pro-apoptotic gene Bax was slightly, but significantly elevated in DCD- versus LD kidneys. Expression of the anti-apoptotic gene Bcl-2 was down-regulated in DBD- compared to LD kidneys. These results suggest that induction of apoptosis may be due to Bax induction during cardiac death, whereas down-regulation of Bcl-2 may be responsible in DBD donors. We showed that in both post-mortem donors the number of apoptotic cells increases between retrieval and 18 hours of cold storage. Despite the stable gene expression levels, there are cellular changes during cold storage resulting in a higher rate of apoptosis, which may account for the deteriorating graft quality during prolonged CI.

Our data strongly support pre-treatment of the DBD donor as a promising strategy to improve graft function and survival. Indeed, low dose dopamine pre-treatment of the donor before kidney transplantation results in a better post-operative kidney function and reduces the incidence of DGF[21]. To reduce the effect of inflammatory cytokines, pre-treatment with methylprednisolone has been used in deceased donors, which resulted in a better outcome after human liver transplantation[20]. Besides this, induction of supraphysiological levels of the cytoprotective gene HO-1 in DBD donors has a beneficial effect on graft function after transplantation[47]. Our study is limited by the use of healthy, young rats without co-morbidity. In this study, we focused on the influence of CI in different donor types. In future study, the use of a transplantation model is required to explore the effect of reperfusion injury. Extensive histology could give insight at cellular level.

Although rodent models of DBD, DCD and LD are unable to capture the full complexity of human donors they are a tool to dissect the relative contributions of brain death and ischemia to pre-transplant organ damage. Here, we show a massive up-regulation of inflammatory, injury, and cytoprotective genes in DBD kidneys, but not in LD- or DCD kidneys at the time of graft retrieval, which remains stable during preservation. The difference between DBD- and DCD donors in inflammatory gene expression may

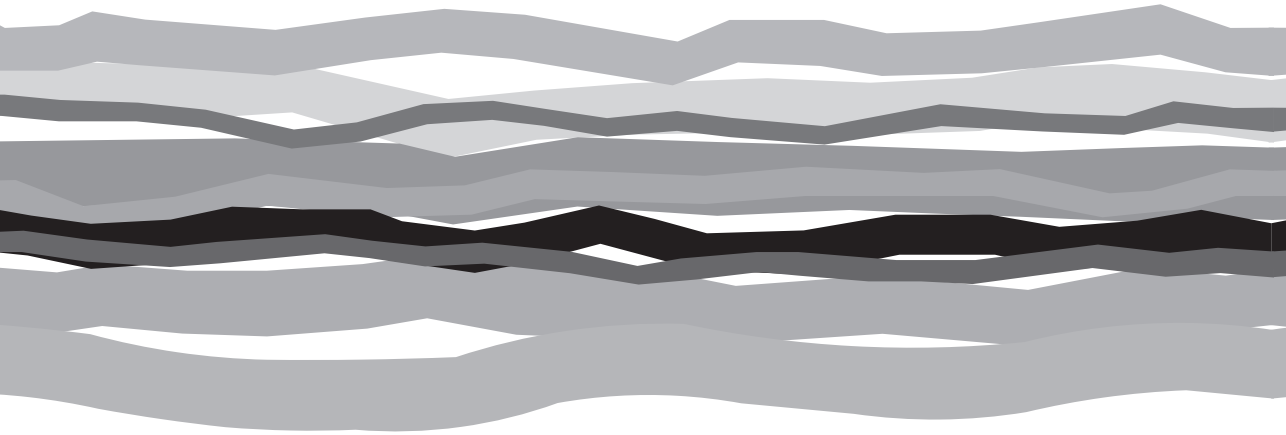
underlie the better outcome after DGF in DCD kidneys[53], and lends further support to the anti-inflammatory treatment of DBD donors[20,21].

References

1. Gjertson DW, Cecka JM. Living unrelated donor kidney transplantation. Kidney Int 2000;58:491-9.
2. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 1995;333:333-6.
3. Chkhotua AB, Klein T, Shabtai E, et al. Kidney transplantation from living-unrelated donors: comparison of outcome with living-related and cadaveric transplants under current immunosuppressive protocols. Urology 2003;62:1002-6.
4. Sanchez-Fructuoso AI, Prats D, Torrente J, et al. Renal transplantation from non-heart beating donors: a promising alternative to enlarge the donor pool. J Am Soc Nephrol 2000;11:350-8.
5. Schuurs TA, Morariu AM, Ottens PJ, et al. Time-dependent changes in donor brain death related processes. Am J Transplant 2006;6:2903-11.
6. Westendorp WH, Leuvenink HG, Ploeg RJ. Brain death induced renal injury. Curr Opin Organ Transplant 2011;16:151-6.
7. Susa D. Mechanisms of ageing in chronic allograft nephropathy. Journal of Organ Dysfunction 2007;3:7-17.
8. Barklin A. Systemic inflammation in the brain-dead organ donor. Acta Anaesthesiol Scand 2009;53:425-35.
9. Weber M, Dindo D, Demartines N, Ambuhl PM, Clavien PA. Kidney transplantation from donors without a heartbeat. N Engl J Med 2002;347:248-55.
10. Wijnen RM, Booster MH, Stubenitsky BM, de Boer J, Heineman E, Kootstra G. Outcome of transplantation of non-heart-beating donor kidneys. Lancet 1995;345:1067-70.
11. Nagaraja P, Roberts GW, Stephens M, et al. Influence of Delayed Graft Function and Acute Rejection on Outcomes After Kidney Transplantation From Donors After Cardiac Death. Transplantation 2012.
12. Barlow AD, Metcalfe MS, Johari Y, Elwell R, Veitch PS, Nicholson ML. Case-matched comparison of long-term results of non-heart beating and heart-beating donor renal transplants. Br J Surg 2009;96:685-91.
13. Metcalfe MS, Butterworth PC, White SA, et al. A case-control comparison of the results of renal transplantation from heart-beating and non-heart-beating donors. Transplantation 2001;71:1556-9.
14. Baan CC, Peeters AM, Demmers MW, et al. FoxP3 T Cells and the Pathophysiologic Effects of Brain Death and Warm Ischemia in Donor Kidneys. Clin J Am Soc Nephrol 2012;7:1481-9.
15. Arnau A, Rodrigo E, Minambres E, et al. Prediction of kidney transplant outcome by donor quality scoring systems: expanded criteria donor and deceased donor score. Transplant Proc 2012;44:2555-7.
16. Snoeijs MG, Schaubel DE, Hene R, et al. Kidneys from donors after cardiac death provide survival benefit. J Am Soc Nephrol 2010;21:1015-21.
17. Bouma HR, Ploeg RJ, Schuurs TA. Signal transduction pathways involved in brain death-induced renal injury. Am J Transplant 2009;9:989-97.
18. Schuurs TA, Gerbens F, van der Hoeven JA, et al. Distinct transcriptional changes in donor kidneys upon brain death induction in rats: insights in the processes of brain death. Am J Transplant 2004;4:1972-81.
19. Kusaka M, Kuroyanagi Y, Kowa H, et al. Genomewide expression profiles of rat model renal isografts from brain dead donors. Transplantation 2007;83:62-70.

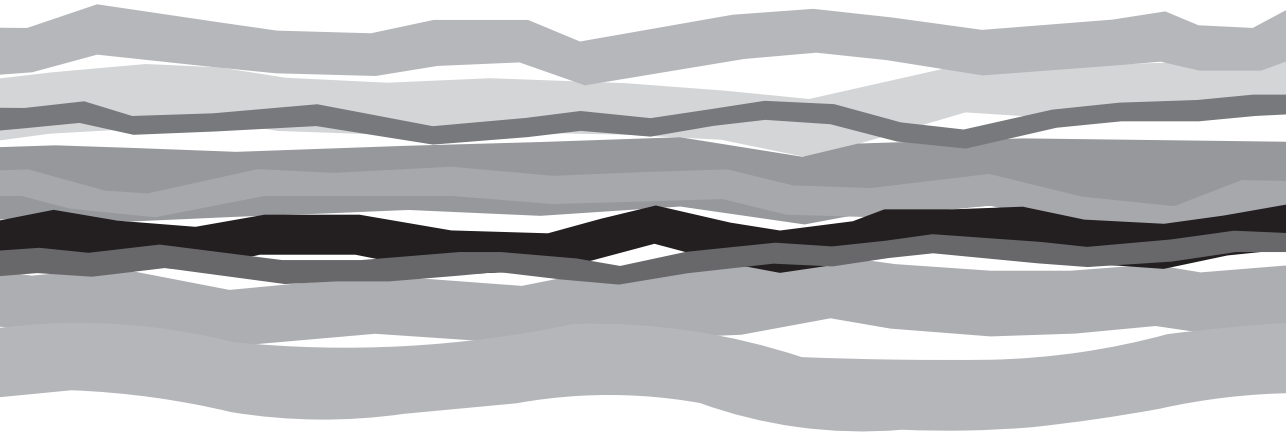
20. Kotsch K, Ulrich F, Reutzel-Selke A, et al. Methylprednisolone therapy in deceased donors reduces inflammation in the donor liver and improves outcome after liver transplantation: a prospective randomized controlled trial. Ann Surg 2008;248:1042-50.
21. Schnuelle P, Gottmann U, Hoeger S, et al. Effects of donor pretreatment with dopamine on graft function after kidney transplantation: a randomized controlled trial. Jama 2009;302:1067-75.
22. Kuecuek O, Mantouvalou L, Klemz R, et al. Significant reduction of proinflammatory cytokines by treatment of the brain-dead donor. Transplant Proc 2005;37:387-8.
23. Plurad DS, Bricker S, Falor A, Neville A, Bongard F, Putnam B. Donor hormone and vasopressor therapy: closing the gap in a transplant organ shortage. J Trauma Acute Care Surg 2012;73:689-94.
24. Laskowski IA, Pratschke J, Wilhelm MM, et al. Early and late injury to renal transplants from non-heart-beating donors. Transplantation 2002;73:1468-73.
25. Nijboer WN, Schuurs TA, van der Hoeven JA, et al. Effects of brain death on stress and inflammatory response in the human donor kidney. Transplant Proc 2005;37:367-9.
26. Nijboer WN, Schuurs TA, van der Hoeven JA, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation 2004;78:978-86.
27. van den Eijnden MM, Leuvenink HG, Ottens PJ, et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp Clin Transplant 2003;1:85-95.
28. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc 1995;27:2893-4.
29. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
30. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int 2004;65:713-8.
31. van der Vliet JA, Warle MC, Cheung CL, Teerenstra S, Hoitsma AJ. Influence of prolonged cold ischemia in renal transplantation. Clin Transplant 2011;25:E612-6.
32. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. Am J Transplant 2011;11:2279-96.
33. Le Dinh H, Monard J, Delbouille MH, et al. A More Than 20% Increase in Deceased-Donor Organ Procurement and Transplantation Activity After the Use of Donation After Circulatory Death. Transplant Proc 2013.
34. Ott U, Aschoff A, Funfstuck R, Jirikowski G, Wolf G. DNA fragmentation in acute and chronic rejection after renal transplantation. Transplant Proc 2007;39:73-7.
35. Straatsburg IH, Abrahamse SL, Song SW, Hartman RJ, Van Gulik TM. Evaluation of rat liver apoptotic and necrotic cell death after cold storage using UW, HTK, and Celsior. Transplantation 2002;74:458-64.
36. Sulikowski T, Domanski L, Zietek Z, et al. The effect of preservation solutions UW and EC on the expression of renin I, angiotensinogen and angiotensin I-converting enzyme genes in rat kidney. Ann Transplant 2011;16:108-13.
37. Hoeger S, Lueg G, Tsagogiorgas C, et al. UW is superior compared with HTK after prolonged preservation of renal grafts. J Surg Res 2011;170:e149-57.
38. Birks EJ, Owen VJ, Burton PB, et al. Tumor necrosis factor-alpha is expressed in donor heart and predicts right ventricular failure after human heart transplantation. Circulation 2000;102:326-31.

39. Murugan R, Venkataraman R, Wahed AS, et al. Increased plasma interleukin-6 in donors is associated with lower recipient hospital-free survival after cadaveric organ transplantation. Crit Care Med 2008;36:1810-6.
40. McKeating EG, Andrews PJ, Signorini DF, Mascia L. Transcranial cytokine gradients in patients requiring intensive care after acute brain injury. Br J Anaesth 1997;78:520-3.
41. Wu H, Ma J, Wang P, et al. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010;21:1878-90.
42. Kruger B, Krick S, Dhillon N, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proc Natl Acad Sci U S A 2009;106:3390-5.
43. Tsung A, Klune JR, Zhang X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. J Exp Med 2007;204:2913-23.
44. Kamo N, Ke B, Ghaffari AA, Busuttil RW, Cheng G, Kupiec-Weglinski JW. The ASC/Caspase-1/IL-1beta signaling triggers inflammatory responses by promoting HMGB1 induction in liver ischemia-reperfusion injury. Hepatology 2013.
45. Metcalfe MS, White SA, Saunders RN, et al. Long-term results of renal transplantation using organs from non-heart-beating donors. Transplant Proc 2001;33:826.
46. Brook NR, White SA, Waller JR, Veitch PS, Nicholson ML. Non-heart beating donor kidneys with delayed graft function have superior graft survival compared with conventional heart-beating donor kidneys that develop delayed graft function. Am J Transplant 2003;3:614-8.
47. Kotsch K, Francuski M, Pascher A, et al. Improved long-term graft survival after HO-1 induction in brain-dead donors. Am J Transplant 2006;6:477-86.
48. Wagner M, Cadetg P, Ruf R, Mazzucchelli L, Ferrari P, Redaelli CA. Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts. Kidney Int 2003;63:1564-73.
49. Nath KA. Heme oxygenase-1: a provenance for cytoprotective pathways in the kidney and other tissues. Kidney Int 2006;70:432-43.
50. Lemos FB, Ijzermans JN, Zondervan PE, et al. Differential expression of heme oxygenase-1 and vascular endothelial growth factor in cadaveric and living donor kidneys after ischemia-reperfusion. J Am Soc Nephrol 2003;14:3278-87.
51. Megyesi J, Andrade L, Vieira JM, Jr., Safirstein RL, Price PM. Coordination of the cell cycle is an important determinant of the syndrome of acute renal failure. Am J Physiol Renal Physiol 2002;283:F810-6.
52. Susa D, Mitchell JR, Verweij M, et al. Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. Aging Cell 2009;8:192-200.
53. White SA, Jain S, Absalom H, et al. Influence of delayed graft function in renal transplants from cadaveric or non-heart-beating donors. Transplant Proc 2000;32:189.



Chapter 4

Inflammatory genes in rat livers from cardiac- and brain death donors



Tanja C. Saat, Denis Susa, Niels F.M. Kok, Sandra van den Engel, Henk P. Roest, Luc J.W. van der Laan, Jan N.M. IJzermans, Ron W.F. de Bruin

Journal of Surgical Research

Abstract

Liver transplantation (LT) is the only life-saving treatment for patients with end-stage liver disease. The increase in patients has prompted the use of not only Donation after Brain Death (DBD) donors but also Living Donors (LD) and Donation after Circulatory Death (DCD) donors. Donor-type affects early graft function and graft survival as evidenced by an increased risk of developing Ischemic Type Biliary Lesions and higher risk of graft loss in DCD- as compared to DBD grafts. Using a rat model, we used qRT-PCR to examine expression levels of pro-inflammatory, cytoprotective and injury genes and determined apoptosis in DCD- and DBD livers at different time-points after retrieval. After retrieval, early mediators of inflammation MCP-1, HMGB1 and TLR4 were increased in DCD livers, whereas the pro-inflammatory genes IL-6, IL-1β, TNF-α, P-selectin and E-selectin were massively up-regulated in DBD- compared to LD livers. HO-1 was increased in both post-mortem groups. After cold ischemia, DCD livers showed increased levels of MCP-1, TLR4 and HMGB1, whereas expression of pro-inflammatory genes in DBD liver remained high. During 12 hours of cold storage, expression levels remained stable except HIF-1α and HMGB1. DCD livers showed higher number of apoptotic cells compared with DBD livers. Compared to livers from LD, DCD livers showed only mild up-regulation of inflammatory markers, but increased levels of MCP-1, HMGB1 and TLR4, and more apoptotic cells. In contrast, DBD livers showed a massive inflammatory response. These differences in tissue injury and inflammatory response might be relevant for the outcome after LT.

Introduction

Liver transplantation (LT) is the only curative treatment for patients with end-stage liver disease. Over the years the outcome after LT has been improved, and this has resulted in more patients eligible for a LT. LT is limited due to the shortage of liver grafts and the increase in patients on the waiting list[1,2]. In an attempt to overcome this discrepancy, the donor pool is being enlarged by using not only Donation after Brain Death (DBD) donors but also Living Donors (LD) and non-heart-beating donors also known as Donation after Circulatory Death (DCD) donors[3,4].

LD livers are retrieved from healthy people and have experienced minimal warm and cold ischemia, which results in decreased inflammation in the transplanted liver[5]. Experience with LD transplantation procedure plays an important role in graft and recipient survival and influences the risk of surgical complications for the donor[5]. Organs from DBD donors suffer from physiological perturbations like severe hemodynamic changes, hormonal changes, electrolyte imbalances, pulmonary changes, hypothermia and inflammation which can contribute to organ damage and inferior post-transplant outcome[6-10]. In DCD donors, death is based on irreversible loss of cardio-pulmonary function. Thus, in contrast to organs from DBD donors, organs from DCD donors are exposed to a period of hypotension, hypoxia and prolonged warm ischemia time, which affects early graft function as well as graft survival[11-13]. DCD liver grafts have an almost 11 times increased risk of developing Ischemic Type Biliary Lesions and 1.85 higher risk of graft loss as compared to DBD grafts[12,14], leading to a higher number of graft failures, re-transplantations and decreased patient survival[11,13].

The process of brain death is relatively well described in literature[6,7,9] and strategies to optimize the organ quality of brain death donors are emerging[15,16]. In contrast, experimental studies comparing perioperative gene expression profiles in liver grafts from LD, DBD- and DCD donors are sparse. Studies are limited to a small number of time points, and a relatively small time span[10], and focused on results after transplantation. Usually 't=0 biopsies' are obtained at the end of the cold storage period, and are unable to take into account the major pathophysiological perturbation that occur during brain death, cardiac arrest, recovery of organs and the cold storage period. Comparative studies in organs from LD, DBD- and DCD donors are currently lacking, and the molecular mechanisms that may account for the differences in transplant outcome are unclear[15,16].

The aim of this study therefore was to compare gene expression profiles of inflammatory, cytoprotective and injury genes in livers from LD, DBD- and DCD donors at the time of retrieval, after clinically relevant cold ischemia times, and over a time-course during cold storage.

Materials and methods

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe. Male Brown Norway rats of 12-14 weeks old, weighing 250–300g, were purchased from Harlan-CPB (Austerlitz, The Netherlands). Rats were randomly assigned to a LD, DCD or DBD group (n=7/group). The LD group served as control for both post-mortem groups. After explantation, livers were stored in University of Wisconsin (UW) solution. At 0, 2, 4, 6 and 12 hours the liver tissue samples were collected and snap frozen in liquid nitrogen until further use. mRNA expression levels in LD, DBD and DCD livers were examined by qRT-PCR and compared at, and between different preservation times. Time-point 0 was defined as the time directly after retrieval. Clinically relevant cold ischemia times were defined as 2 hours in the LD group and 12 hours in the DBD and DCD groups. During cold storage mRNA expression levels were determined at 0, 2, 4, 6 and 12 hours. In each group mRNA expression levels were compared with the previous time-point to determine possible changes during cold storage. For immunohistochemistry DCD- and DBD livers were used directly after retrieval and after 12 hours of cold ischemia and stained with cleaved caspase-3.

Experimental models

Animals were anesthetized with isoflurane. After intubation, anesthesia was maintained using a mixture of N_2O/O_2/2% isoflurane. Animals were pressure control ventilated on a Siemens Servo 900C ventilator (Maqet Critical Care AB, Solna, Sweden) with 14 cm H_2O PIP, 4 cm H_2O PEEP with a frequency of 40 breaths per minute. In the DBD group a frontolateral trepanation was made and a balloon catheter (Fogarty Arterial Embolectomy Catheter: 5F, Baxter Healthcare Co., Irvine, Ca, USA) was introduced in the extradural space and slowly inflated, causing a gradually increasing intra-cranial pressure[17]. Herniation of the brainstem and brain death was confirmed by dilated and fixed pupils, the absence of corneal reflexes, and an apnoea test. DBD animals received ventilation but no further anaesthesia. Intra-arterial pressure was continuously monitored via a PE50 catheter placed in the carotid artery. Only rats with stable mean arterial pressure (MAP > 80 mm Hg) during the 6 hours of brain death were included in the experiment. After 6 hours the liver was removed, flushed with PBS and stored in UW solution at 4°C. In the DCD group cardiac arrest was induced by isoflurane overdose. The cardiac arrest period started when the blood pressure had dropped to 5-7 mm Hg, and lasted 20 minutes, the time commonly needed before starting with cold storage of the liver. The DCD donors are type III of the Maastricht criteria[18]. In the LD group, livers were collected after one hour of mechanical ventilation using an N_2O/O_2/2% isoflurane mixture.

mRNA expression analysis

Liver sections were homogenized for 30 seconds using a pro 200 homogenizer equipped (PRO scientific inc., Oxford, CT) in 1.0 ml Trizol (Invitrogen, The Netherlands). Total RNA was isolated according to the manufacturer's guidelines of Trizol (Invitrogen), precipitated, and subsequently dissolved in DEPC-treated water. RNA concentration was determined using a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop technologies, Wilmington, DE) and stored at -80°C until further use.

For cDNA synthesis, approximately 1 μg of total RNA was used as template for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol with 250 ng of random hexamers (Promega, The Netherlands) as a starting point for reversed transcription and 40 IU RNAseOUT (Invitrogen) to prevent RNA degradation. All temperature-dependent steps were performed using a Biometra T-gradient cycler (Biometra Gmbh, Göttingen, Germany). cDNA samples were stored at -20°C.

Genes

As genes indicative for inflammation we used TLR4, HMGB1, IL-1β, IL-6, TNF-α, MCP-1, P-selectin and E-selectin. To assess cytoprotection we measured HO-1, VEGF, and HIF-α. P21, a cell cycle inhibitor, and the apoptosis markers Bax and Bcl-2 were used as a markers indicative for liver injury. Primers used to amplify the genes of interest were obtained from Sigma, the Netherlands, **Table 1.**

qRT-PCR analysis

For quantitative analysis of transcription, 0.33 μl of cDNA was amplified using a BioRad Icycler (Bio-Rad laboratories). Reaction mixtures of 25 μl contain cDNA, 5 μmol of each primer, 0.5 U Platinum Taq (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTP each, and 0.075 μl of a 200x stock SYBR Green I solution (Sigma). Cycling conditions consisted of 3 minutes activation at 95°C, 40 cycles of 15" 95°C, 30" 60°C, and 30" 72°C, followed by 1' 95°C and 1' 65°C. Samples were subsequently subjected to a melt curve analysis with a temperature range of 60-94.5°C to verify amplified products.

ΔCt values of genes of interest were calculated as described by Pfaffl[19] using β-actin as a reference gene. To compare all individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group. The LD group at 0 hour was used in the 0 hour comparison (**Figure 2A, 2B**), the LD group at 2 hours was used in the 2 hour versus 12 hour comparison (**Figure 1A, 1B**), and the respective time points in the time course (**Figure 3**). Subsequently the fold increase was calculated using the Pfaffl equation: $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM.

Table 1. Primers used for qRT-PCR.

Housekeeping Gene	Forward Primer	Reverse Primer
β-actin	'5-gacccagatcatgtttgagacc-3'	'5-gatgggcacagtgtgggtgac-3'
Inflammatory Genes	**Forward Primer**	**Reverse Primer**
IL-1β	'5-gaggctgacagaccccaaaaga-3'	'5-tccacagccacaatgagtgaca-3'
IL-6	'5-gtctcgagcccaccaggaac-3'	'5-agggaaggcagtggctgtca-3'
TNF-α	'5-gaccctcacactcagatcatcttct-3'	'5-tgctacgacgtgggctacg-3'
MCP-1	'5-gccatcagcccacaggtgtt-3'	'5-gggacactggctgcttgtga-3'
P-selectin	'5-aagatggtcagcgcctccac-3'	'5-atcgaaccgatgggacagga-3'
E-selectin	'5-cccacatgtgcaggggtaca-3'	'5-tggcccactgcaactcatgt-3'
TLR4	'5-tccgctggttgcagaaaatg-3'	'5-tcccagccagatgcaagaga-3'
HMGB1	'5-ccgccaagctgaaggagaag-3'	'5-ccctttttcgctgcatcagg-3'
Cytoprotective Genes	**Forward Primer**	**Reverse Primer**
HO-1	'5-tttcagaagggtcaggtgtcca-3'	'5-agtagagcggggcatagactgg-3'
p21	'5-ccacagcgatatcgagacactca-3'	'5-acagacgacggcatactttgctc-3'
Injury Genes	**Forward Primer**	**Reverse Primer**
HIF-1α	'5-agtcagcaacgtggaaggtgc-3'	'5-acaaatcagcaccaagcacgtc-3'
VEGF	'5-tgcactggaccctggctttac-3'	'5-ttctgctccccttctgtcgtg-3'
Bax	'5-tgctgatggcaacttcaactgg-3'	'5-tggttctgatcagctcgggca-3'
Bcl-2	'5-acatcgccctgtggatgactg-3'	'5-gcatgctggggccatatagttc-3'

Immunohistochemistry

Frozen sections, 5μm-thick, were stained with a polyclonal antibody against cleaved caspase-3 (Asp175; Cell Signaling), an early apoptotic marker. Cleaved caspase-3 was diluted 1:300 and visualized with a horseradish peroxidase-conjugated secondary antibody (dilution 1:500, goat-anti-rabbit IgG/HRP, DAKO, Denmark). The number of positive cells was determined in 10 high power fields at a magnification of 200x by 2 independent observers blinded to the treatment.

Statistical Analysis

Data were analysed using a Kruskal Wallis ANOVA, followed by a Mann-Whitney–U test. Analysis was performed using SPSS v20.0 for Windows. A p value ≤0.05 was considered statistically significant.

Results

Gene expression profiles directly after retrieval

To study changes in gene expression levels between the moment of liver graft procurement and the end of the cold storage period, we analysed gene expression levels at

the time of retrieval of the livers (t=0) (**Figure 1A**). In DBD livers, the pro-inflammatory genes IL-1β, IL-6 and TNF-α were 14.8-fold (p=0.003), 326.5-fold (p=0.003), and 22.5-fold (p=0.004) increased compared with LD livers. P-selectin (41.7-fold, p=0.004) and E-selectin (12.9-fold, p=0.037) were also increased in DBD livers, whereas TLR4 expression was decreased (p=0.001). In DCD livers, only HMGB1 was significantly increased (p=0.009) compared to LD livers. HO-1 was increased in both post-mortem groups, 5.6-fold in DCD (p=0.021) and 12.6 fold in DBD livers (p=0.003) compared with the LD group (**Figure 1B**). In DCD livers, HIF-1α was 2.6-fold increased. VEGF was 0.3 fold down-regulated in DBD livers (p=0.004). In DBD livers, Pro-apoptotic Bax was decreased 0.5-fold (p=0.021) whereas anti-apoptotic Bcl-2 expression was unchanged. In DCD livers Bax expression was not altered, but Bcl-2 showed a 6.9-fold increase (p=0.001). Cell cycle inhibitor p21 showed no significant difference.

In conclusion, compared to LD, DCD livers showed only mild up-regulation of inflammatory markers, but increased levels of MCP-1, HMGB1 and TLR4, and more apoptotic cells. DBD livers showed a massive up-regulation of pro-inflammatory, cytoprotective and injury genes.

Gene expression profiles after cold ischemia

Following 12 hours of cold ischemia, expression levels of the pro-inflammatory genes IL-1β, IL-6 and TNF-α, and P-selectin and E-selectin, largely reflected those found directly after recovery in DBD livers (**Figure 2A**). In DCD livers, only IL-6 expression was slightly but significantly increased (4.6-fold,p=0.018) compared to LD livers. While there was no difference after recovery, MCP-1 was increased 30.8-fold (p=0.016) in the DCD- but slightly down-regulated in DBD livers (p=0.017). E-selectin was increased in DCD livers 4.1-fold (p=0.011). HMGB1 expression showed a similar trend as TLR4 in DCD livers. DBD livers showed a down-regulation of TLR4 (p=0.008), which is comparable with the results found after recovery.

In DBD livers HO-1 was 16.9-fold (p=0.021) increased, while DCD livers showed no significant difference compared to LD livers (**Figure 2B**). HIF-1α showed no significant changes in the post-mortem livers. VEGF and Bax showed a decrease of 0.2-fold (p=0.008) and 0.4-fold (p=0.006) respectively in the DBD livers. In DCD livers, Bcl-2 showed an up-regulation of 4.6-fold (p=0.009). In both post-mortem groups, p21 up-regulation reflected that at recovery, but reached significance after 12 hours of cold preservation. These data show that biological processes in the different donors are a major determinant of gene expression in the graft prior to transplantation.

Gene expression profiles during 12 hours of cold storage

Since the expression levels of some genes changed between the time of recovery of the organ and the end of the cold storage period, we addressed when changes in gene-

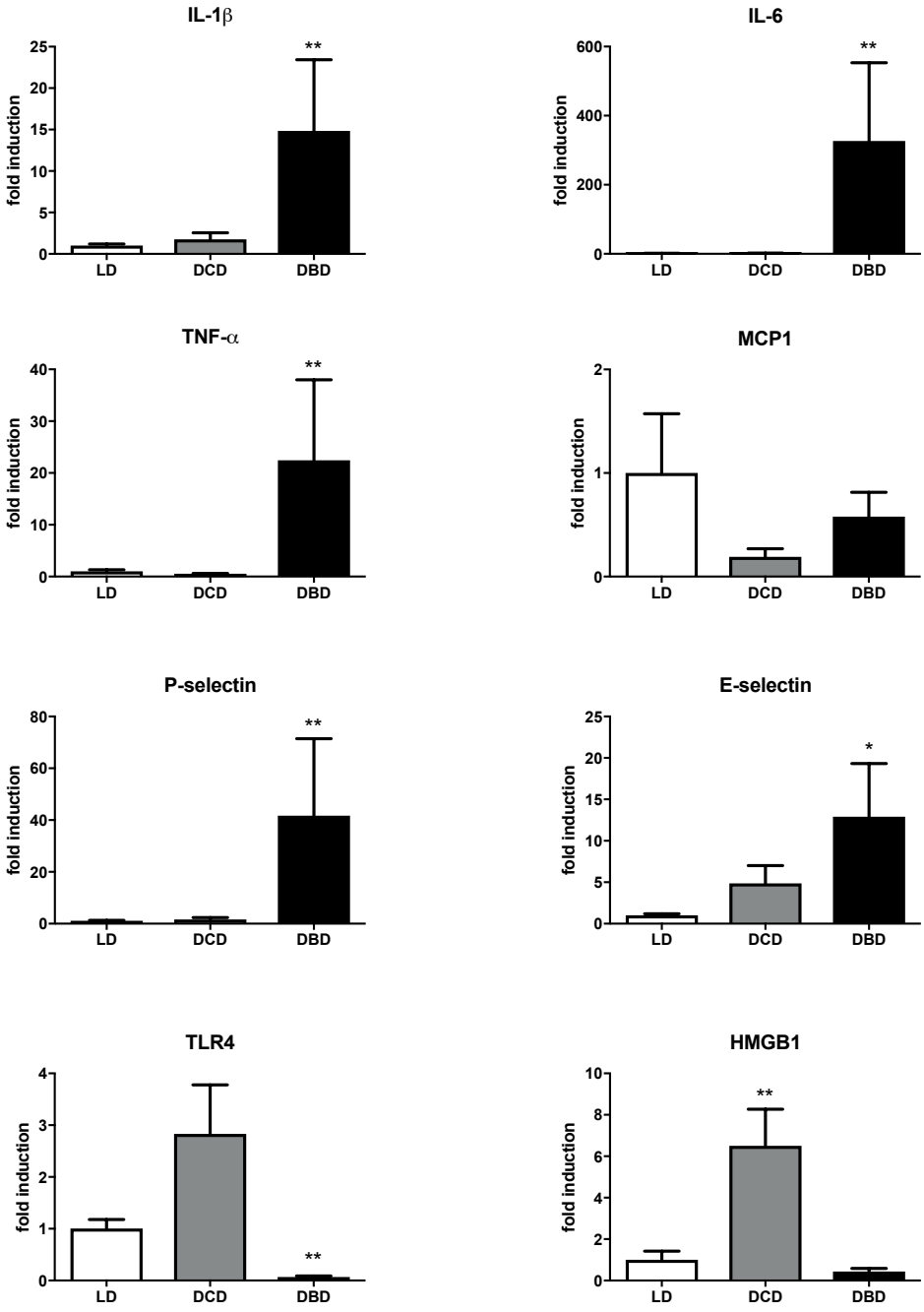


Figure 1A. Gene expression profiles of inflammatory genes directly after recovery. DBD livers showed an up-regulation of the inflammatory genes compared to the LD. DCD livers showed mild inflammation but an up-regulation of HMGB1 compared to LD livers. mRNA expression levels were determined by qRT-PCR directly after recovery of the liver. To compare the individual expression levels of each animal, ΔCt values

Figure 4.1 continued
were normalized to the average ΔCt of the LD group at time point 0. Subsequently the fold increase was calculated using $2^{-\Delta\Delta Ct}$. Results are expressed as mean ± SEM (n=7/group). *p≤0.05, **p≤0.01 compared to LD.

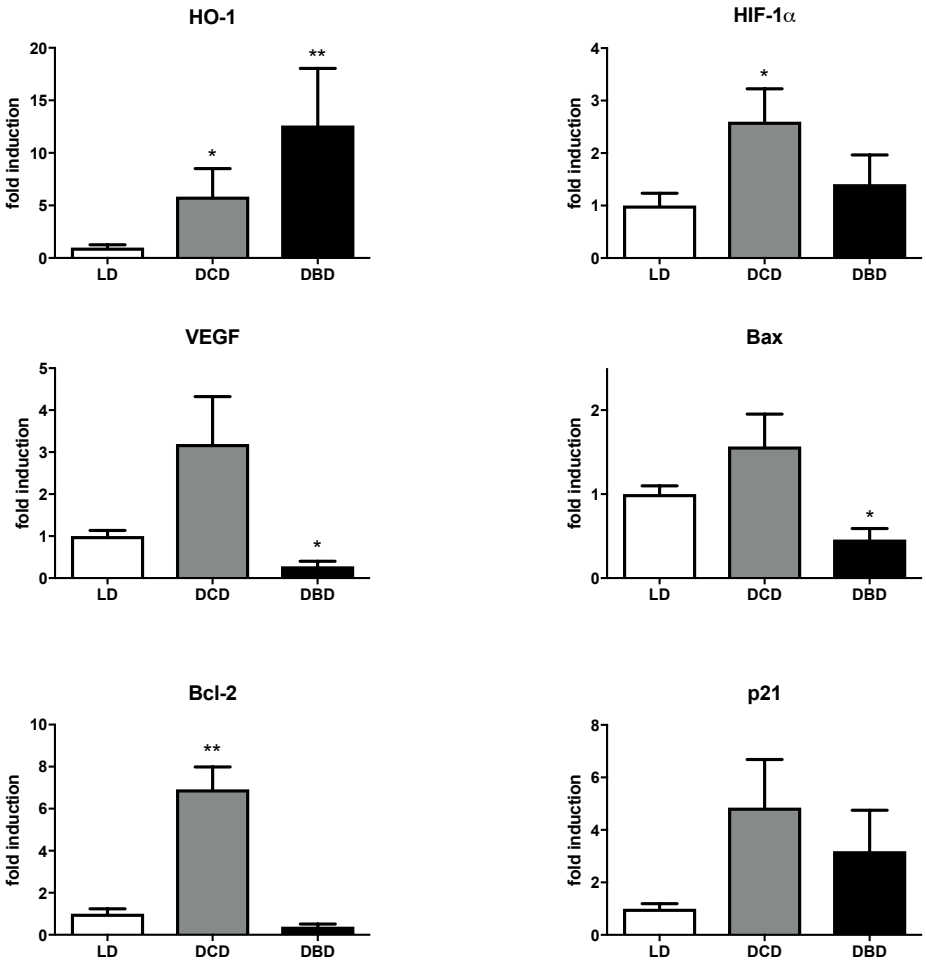


Figure 1B. Expression levels of cytoprotective genes directly after recovery. HO-1 was up-regulated in both DBD- and DCD donors compared to LD. HIF1-α was up-regulated in the DCD livers compared to LD livers. VEGF was down-regulated in DBD donors compared to LD. In DBD livers, Bax was decreased whereas Bcl-2 expression was unchanged. In DCD livers, Bax expression was not altered, but Bcl-2 showed an increase. Results are expressed as mean ± SEM (n=7/ group). *p≤0.05, **p≤0.01 compared to LD.

expression would occur during cold storage. Gene expression of HO-1, HIF-1α, Bcl-2, Bax, VEGF (not shown), TLR4 and HMGB1 were examined after 0, 2, 4, 6 and 12 hours of cold ischemia (**Figure 3**).

In LD livers all the expression levels remained stable during cold preservation. In both post-mortem groups, HO-1, VEGF (not shown), and Bax mRNA expression levels did

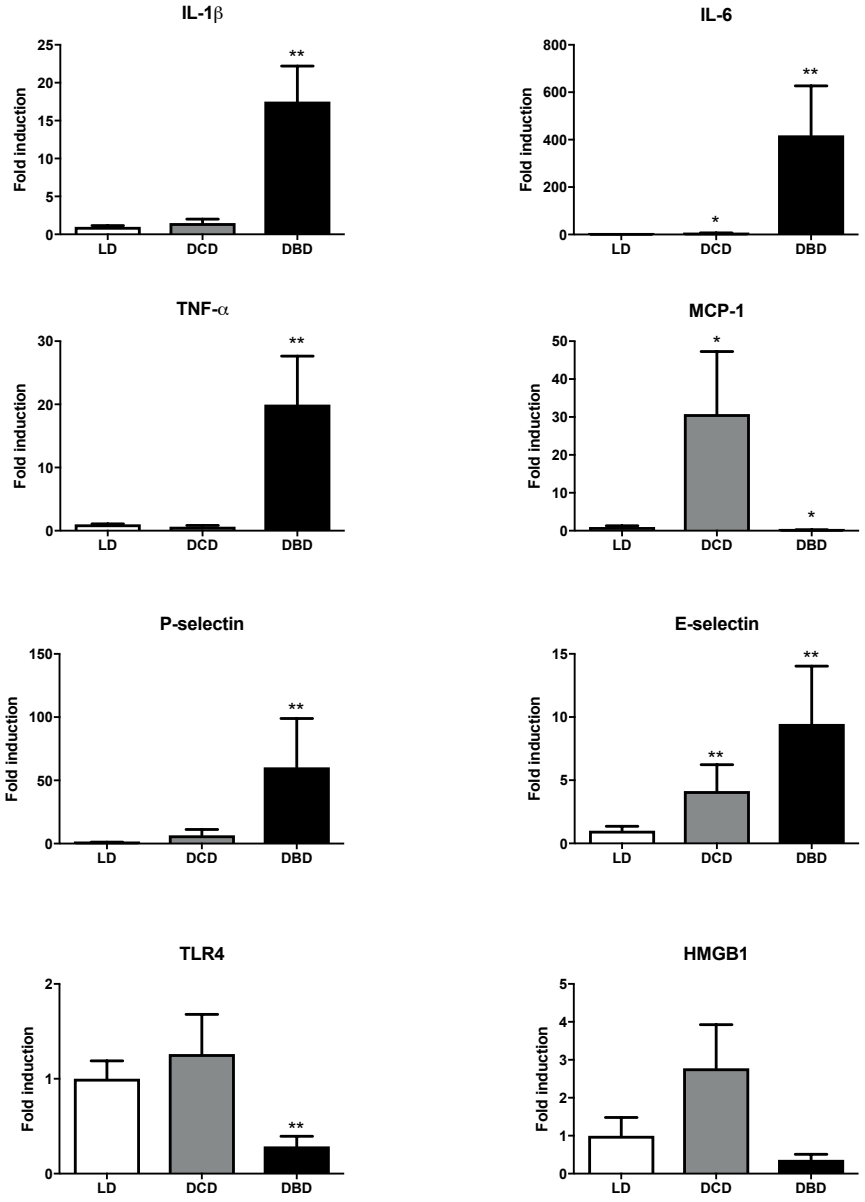


Figure 2A. Gene expression profiles of inflammatory genes in the liver after clinically relevant CI times. mRNA expression levels of genes indicative of inflammation were determined by qRT-PCR after 2 hours of CI in LD, and after 12 hours of CI in DBD- and DCD donors. DBD donors showed an up-regulation of the inflammatory genes in DBD livers, while DCD livers showed mild inflammation compared to LD. Interestingly, MCP-1 was increased in the DCD- but slightly down-regulated in DBD- compared to LD livers. To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at 2 hours. Subsequently the fold increase was calculated using $2^{-\Delta\Delta Ct}$. Results are expressed as mean ± SEM (n=7/group). *p≤0.05, **p≤0.01 compared to LD.

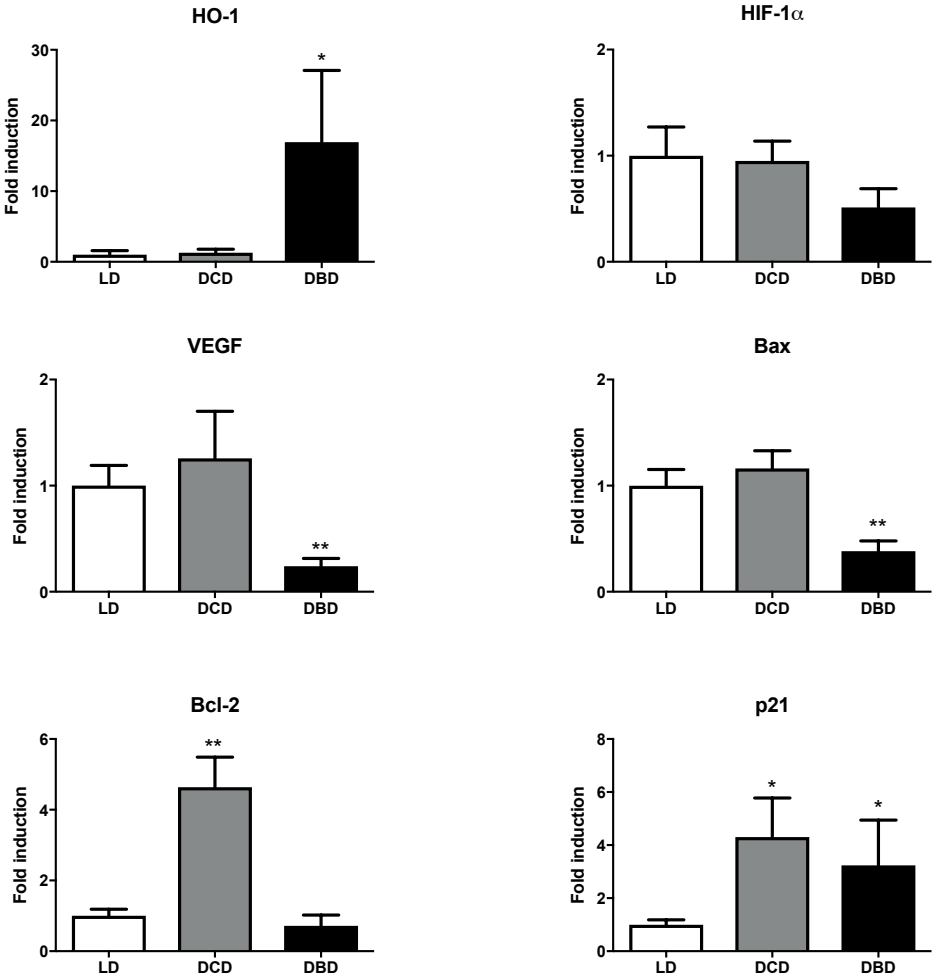


Figure 2B. Gene expression profiles of cytoprotective and injury genes in the liver after CI times. Expression of the cytoprotective gene HO-1 was increased in DBD livers compared to LD livers. mRNA expression levels of genes indicative of inflammation were determined by qRT-PCR after 2 hours of CI in LD, and after 12 hours of CI in DBD- and DCD donors. Results are expressed as mean ± SEM (n=7/group). *p≤0.05, **p≤0.01 compared to LD.

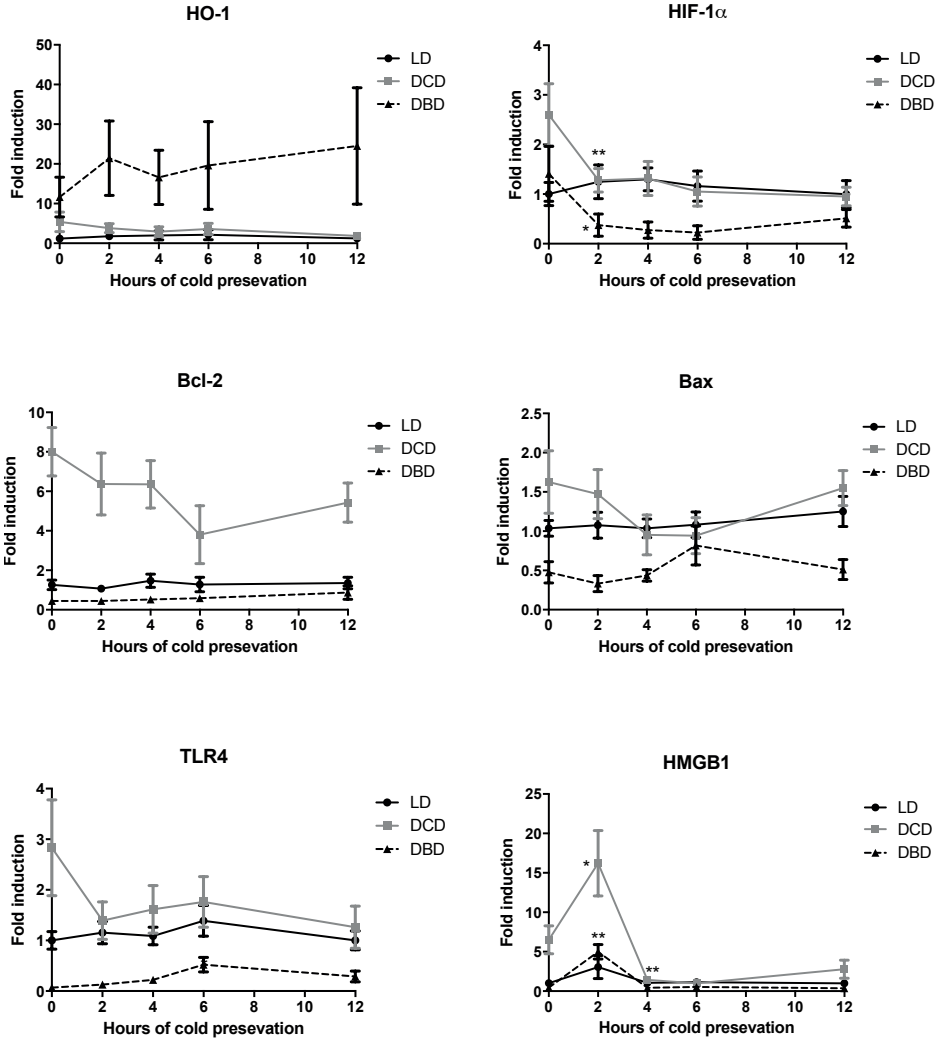


Figure 3. Gene expression profiles during 12 hours of cold storage. Expression levels of HO-1, HIF-1α, Bcl-2, Bax, TLR4 and HMGB1 were determined directly after liver resection, and at 2, 4, 6 and 12 hours of cold preservation in LD-, DCD- and DBD livers. Gene expression levels of HO-1, Bax and VEGF remain stable during cold preservation in LD-, DCD- and DBD livers. In LD livers all the expression levels remain stable during cold preservation. In DCD livers HIF-1α and HMGB1 showed a decrease in expression levels after 2 hours of cold storage. Bcl-2 showed a decrease after 6 hours of cold storage. In DBD livers HIF-1α and HMGB1 showed mild changes. To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at the respective time points. Subsequently the fold increase was calculated using $2^{-\Delta\Delta Ct}$. Results are expressed as mean ± SEM (n=7/group). *p≤0.05, **p≤0.01 compared to LD.

not significantly change between recovery of the organ and 12 hours of cold ischemia. HIF-1α showed a significant decrease in DCD- (p=0.004) and DBD livers (p=0.018) between 0 and 2 hours of cold storage, but remained stable thereafter. In DCD livers, Bcl-2 expression levels showed a decreasing trend between 0 and 6 hours (p=0.018) of cold ischemia. In both DCD- and DBD livers TLR4 levels showed no significant difference over time. In both post-mortem groups HMGB1 levels showed a similar trend. HMGB1 levels were significantly increased after 2 hours of cold preservation in both DCD- (p=0.035) and DBD livers (p=0.002), returned to levels measured at retrieval, and remained stable thereafter. In conclusion, during cold storage, gene expression profiles remain stable in LD livers, while in DCD- and DBD livers HIF-1α, Bcl-2 and HMGB1 expression levels show mild changes.

Apoptosis during cold storage

DCD- and DBD livers collected directly after retrieval and after 12 hours of cold storage were stained for cleaved caspase-3 (**Figure 4**). In DCD livers the number of apoptotic cells is approximately 4 times higher directly after retrieval compared to DBD livers (p<0.05). In both DCD- and DBD livers the number of apoptotic cells found directly after retrieval does not change during cold preservation.

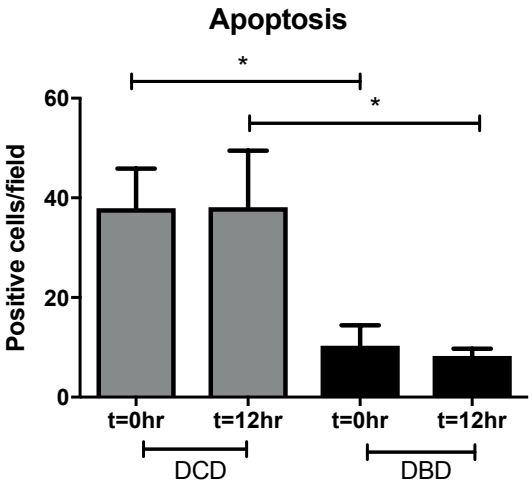


Figure 4. Early apoptosis as determined by cleaved caspase-3 staining in DCD- and DBD livers directly after retrieval and after 12 hours of cold ischemia. DCD livers show significant more apoptotic cells directly after retrieval and after cold ischemia compared with DBD livers. Results are expressed as mean ± SEM (*p<0.05).

Discussion

Our study shows a differential gene expression profile between livers retrieved from DBD- versus DCD donors. Livers from DCD donors show only mild up-regulation of inflammatory genes, but increased expression levels of MCP-1, TLR4, and HMGB1

directly after recovery. In contrast, DBD donors show a massive up-regulation of pro-inflammatory, cytoprotective and injury genes. During 12 hours of cold storage, mRNA gene expression profiles remain stable in LD livers, while DCD- and DBD livers show mild changes in mRNA expression of HIF-1α, Bcl-2 and HMGB1 during cold ischemia. Despite the lower expression of inflammatory genes in DCD- compared to DBD livers, the outcomes after DCD liver transplantation are inferior to those using DBD livers[20]. DCD donors show an increased rate of biliary complications[1,12,13], higher post-transplant morbidity and inferior graft survival[3,21].

The most striking finding in our study is the differential expression of inflammatory genes such as HMGB1, TLR4, MCP-1 IL-1β, IL-6, and TNF-α between DCD- and DBD livers. HMGB1 and TLR4 were highly expressed at the time of recovery and after cold preservation in DCD- but not in DBD livers. HMGB1 is a nuclear transcription factor that can be released from necrotic cells and has been identified as a ligand for TLR4[22]. During hepatic ischemia reperfusion injury (IRI) it acts as a central mediator of TLR4 dependent[23-25] production of pro-inflammatory cytokines[26]. Although HMGB1 can be passively released by necrotic cells[22], it may also be mobilized and released in response to hypoxia[23]. The prolonged warm ischemia time in DCD livers might be the reason why HMGB1 is up-regulated at procurement. HMGB1 levels correlate more with markers of overall graft damage such as aminotransferase than levels of individual cytokines such as TNF-α or IL-6[25].

MCP-1, or CCL-2, is produced by activated hepatocytes[27], cholangiocytes and portal fibroblasts[28]. After binding to the cognate receptor CCR2, MCP-1 attracts monocytes and dendritic cells to sites of tissue injury, and is able to induce apoptosis. MCP-1 is considered an early mediator of inflammation[29], and is implicated in hepatic fibrosis[30]. Over-expression of MCP-1 in the liver leads to inflammation and injury, while MCP-1 deficient mice are protected against toxic liver injury and oxidative stress[31]. In humans high levels of MCP-1 early after transplantation are associated with poor early graft function after LT[32] and it has been suggested that MCP-1 contributes to the development of biliary atresia[33]. The differential expression of early versus late markers of inflammation suggests that the kinetics of injury and inflammation differ between DBD- and DCD livers. During 6 hours of brain death in our model, there is sufficient time to mount an inflammatory response[7-9], whereas in DCD donors (20 minutes of cardiac arrest) this response might still be in an early phase and may reach its full magnitude only after transplantation (**Figure 5**).

High levels of inflammatory cytokines and adhesion molecules in DBD livers are associated with the physiological changes that occur during brain death. These changes lead to organ damage and higher rates of primary non-function post-transplant compared with LD livers[6-9]. The disrupted blood-brain barrier in DBD donors might contribute to the high levels of inflammatory cytokines in DBD donors, since the ischemic brain

releases inflammatory mediators, which provokes a systemic inflammatory response[7,34]. Here we show that it is likely that the liver itself contributes to systemic levels of inflammatory cytokines and systemic inflammation during brain death as well[9].

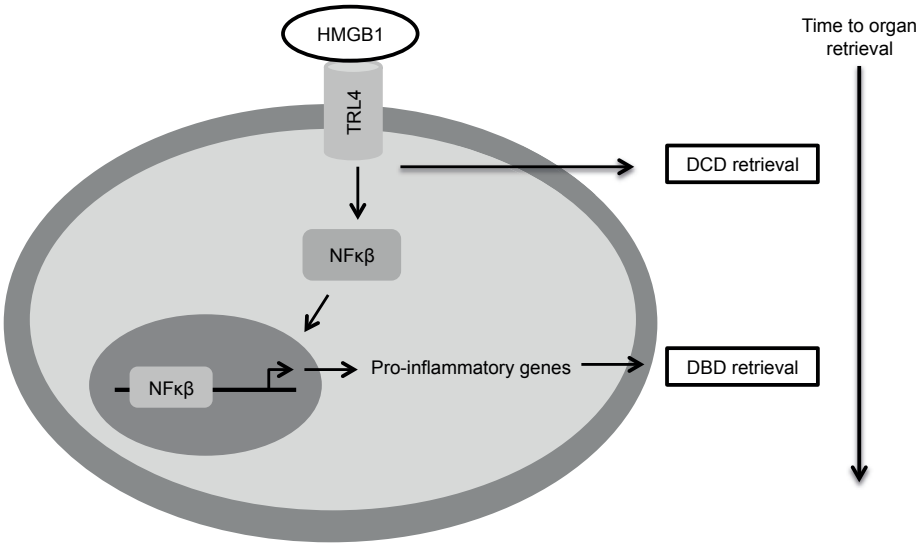


Figure 5. Proposed model for the differential kinetics of the inflammatory response in DCD versus DBD donors. HMGB1, secreted by necrotic cells, is a ligand for TLR4, which activates NFκβ and induces the transcription of inflammatory genes. During brain death there is sufficient time to mount this inflammatory response in the donor, whereas in DCD donors this response might still be in an early phase and may reach its full magnitude only after transplantation of the liver.

The cytoprotective gene HO-1 was significantly up-regulated in both post-mortem groups at the time of procurement, and remained stable during 12 hours of cold preservation. Over-expression of HO-1 in the liver interferes with inflammation, oxidative stress and apoptosis[35], and results in better outcomes after IRI[36,37]. The protection afforded by physiological over-expression of HO-1 in DBD livers is likely insufficient[38], but could be further elaborated in experimental studies. In DCD livers HIF-1α, the transcription factor inducing the production of VEGF, and VEGF are increased at time of retrieval compared to LD livers. DBD livers showed no significant difference in HIF-1α expression, and decreased VEGF expression. The data suggest that during the warm anoxic phase of DCD donation HIF-1α and subsequent VEGF are rapidly induced. Interestingly, the cyclin-dependent kinase inhibitor p21 was also strongly induced in both post-mortem groups. By binding to PCNA and inhibiting its action in DNA replication, p21 is a key regulator of proliferation. Previously we showed that there is a critical, temporal balance between the expression levels of p21 and proliferation of the tubular compartment after renal ischemia[39,40]. The up-regulation of p21, like HO-1, in our study may be an indicator

of liver damage. The pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2 are involved in apoptosis of endothelial cells and hepatocytes after warm- and cold ischemia during LT[41]. The Bax/Bcl-2 ratio shows at the genetic level if there is a pro-apoptotic or anti-apoptotic state. Compared to LD livers, DCD livers showed a Bax/Bcl-2 ratio at explantation of 0.23 and after cold storage of 0.25. In DBD livers the Bax/Bcl2 ratio was 1.18 and after cold storage of 0.53. It is unlikely that these minor differences bear biological significance.

During 12 hours of cold storage, in both DBD- and DCD livers significant differences were found in gene expression level of HIF-1α and HMGB1. The use of UW-solution may reduce apoptosis and hence prevent degradation of RNA[42], whereas the lack of oxygen during cold preservation precludes the transcription of DNA into mRNA. This might explain why, for most genes, mRNA levels remain stable during cold preservation. After excluding technical reasons as cause for the changes in expression levels in the DBD- and DCD group, these differences in results might be explained by the poorer quality of the post-mortem organs. However, a plausible mechanism for the observed changes is currently lacking. Cleaved caspase-3 staining showed that DCD livers have significantly higher numbers of early apoptotic cells compared with DBD livers at graft retrieval. In both DBD- and DCD livers, these numbers do not change during cold storage. The higher numbers of apoptotic cells might explain why DCD livers have a worse outcome after LT, despite the lower expression levels of inflammatory and injury genes.

Our study is limited by the use of experimental models of DBD, DCD and LD, which are unable to capture the full complexity of human donors that differ in age, sex and co-morbidity. Nevertheless, they are a tool to dissect the relative contributions of brain death and ischemia to pre-transplant organ damage. We used whole tissue homogenates and not specifically looked at the biliary tree. Especially DCD donor livers suffer from biliary complications, compared with DBD donor livers[43-45]. Nevertheless, the differential expression levels of inflammatory genes suggest a different postoperative course, which may be related to the different postoperative outcomes.

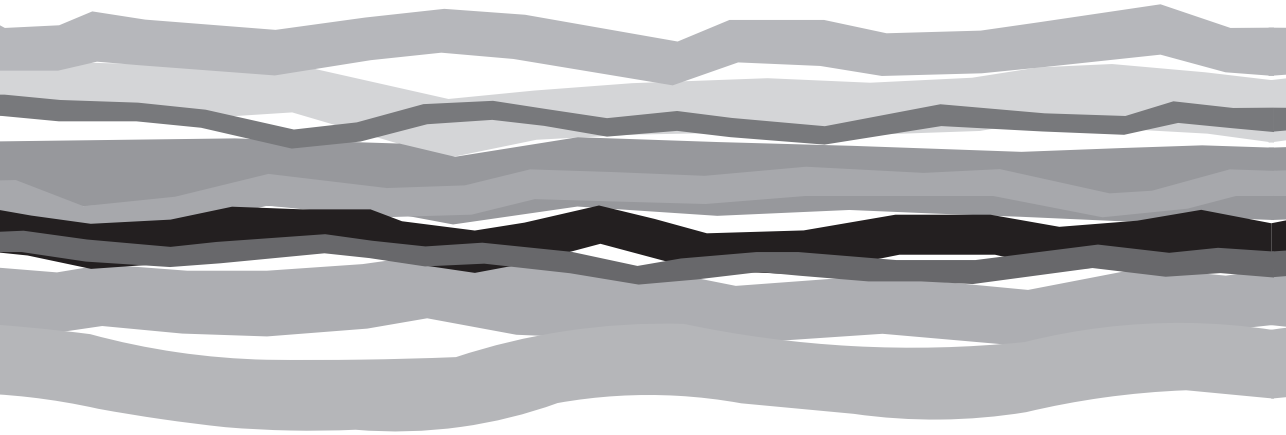
In conclusion, using these models, we show an increase in MCP-1, HMGB1 and TLR4 expression in DCD donors, and a massive up-regulation of inflammatory, cytoprotective, and injury genes in liver transplants from DBD donors, but not from living-, or DCD donors at the time of liver procurement. This suggests that the kinetics of injury and inflammatory processes might be different, as outlined in **Figure 5**, and may be relevant for the different rates and types of complications and graft outcome, and guide more effective early treatment of the recipient based on donor type.

References

1. Foley DP, Fernandez LA, Leverson G, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg 2011;253:817-25.
2. Ciria R, Briceno J, Rufian S, Luque A, Lopez-Cillero P. Donation after cardiac death: where, when, and how? Transplant Proc 2012;44:1470-4.
3. Taner CB, Bulatao IG, Willingham DL, et al. Events in procurement as risk factors for ischemic cholangiopathy in liver transplantation using donation after cardiac death donors. Liver Transpl 2012;18:100-11.
4. Jassem W, Koo DD, Muiesan P, et al. Non-heart-beating versus cadaveric and living-donor livers: differences in inflammatory markers before transplantation. Transplantation 2003;75:1386-90.
5. Kaido T, Uemoto S. Does living donation have advantages over deceased donation in liver transplantation? J Gastroenterol Hepatol 2010;25:1598-603.
6. Floerchinger B, Oberhuber R, Tullius SG. Effects of brain death on organ quality and transplant outcome. Transplant Rev (Orlando) 2012;26:54-9.
7. Barklin A. Systemic inflammation in the brain-dead organ donor. Acta Anaesthesiol Scand 2009;53:425-35.
8. Schuurs TA, Morariu AM, Ottens PJ, et al. Time-dependent changes in donor brain death related processes. Am J Transplant 2006;6:2903-11.
9. Danobeitia JS, Sperger JM, Hanson MS, et al. Early activation of the inflammatory response in the liver of brain-dead non-human primates. J Surg Res 2012;176:639-48.
10. Weiss S, Kotsch K, Francuski M, et al. Brain death activates donor organs and is associated with a worse I/R injury after liver transplantation. Am J Transplant 2007;7:1584-93.
11. Woodside KJ. Donation after cardiac death and liver transplantation. J Surg Res 2012.
12. Le Dinh H, de Roover A, Kaba A, et al. Donation after cardio-circulatory death liver transplantation. World J Gastroenterol 2012;18:4491-506.
13. Monbaliu D, Pirenne J, Talbot D. Liver transplantation using Donation after Cardiac Death donors. J Hepatol 2012;56:474-85.
14. Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. Ann Surg 2006;244:555-62.
15. Gehrau RC, Mas VR, Dumur CI, et al. Regulation of Molecular Pathways in Ischemia-Reperfusion Injury After Liver Transplantation. Transplantation 2013.
16. Conti A, Scala S, D'Agostino P, et al. Wide gene expression profiling of ischemia-reperfusion injury in human liver transplantation. Liver Transpl 2007;13:99-113.
17. van den Eijnden MM, Leuvenink HG, Ottens PJ, et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp Clin Transplant 2003;1:85-95.
18. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc 1995;27:2893-4.
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
20. Mathur AK, Heimbach J, Steffick DE, Sonnenday CJ, Goodrich NP, Merion RM. Donation after cardiac death liver transplantation: predictors of outcome. Am J Transplant 2010;10:2512-9.
21. Dubbeld J, Hoekstra H, Farid W, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97:744-53.

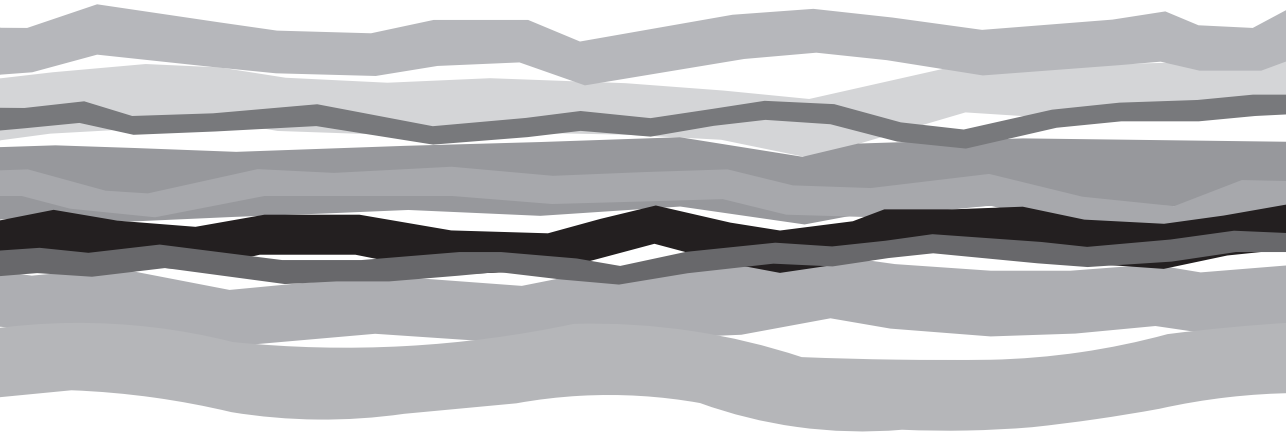
22. Liu A, Fang H, Dirsch O, Jin H, Dahmen U. Oxidation of HMGB1 causes attenuation of its pro-inflammatory activity and occurs during liver ischemia and reperfusion. PLoS One 2012;7:e35379.
23. Tsung A, Klune JR, Zhang X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. J Exp Med 2007;204:2913-23.
24. Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005;201:1135-43.
25. Klune JR, Tsung A. Molecular biology of liver ischemia/reperfusion injury: established mechanisms and recent advancements. Surg Clin North Am 2010;90:665-77.
26. Wu H, Ma J, Wang P, et al. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010;21:1878-90.
27. Dambach DM, Watson LM, Gray KR, Durham SK, Laskin DL. Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. Hepatology 2002;35:1093-103.
28. Kruglov EA, Nathanson RA, Nguyen T, Dranoff JA. Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts. Am J Physiol Gastrointest Liver Physiol 2006;290:G765-71.
29. Ramnath RD, Bhatia M. Substance P treatment stimulates chemokine synthesis in pancreatic acinar cells via the activation of NF-kappaB. Am J Physiol Gastrointest Liver Physiol 2006;291:G1113-9.
30. Seki E, de Minicis S, Inokuchi S, et al. CCR2 promotes hepatic fibrosis in mice. Hepatology 2009;50:185-97.
31. Zamara E, Galastri S, Aleffi S, et al. Prevention of severe toxic liver injury and oxidative stress in MCP-1-deficient mice. J Hepatol 2007;46:230-8.
32. Friedman BH, Wolf JH, Wang L, et al. Serum cytokine profiles associated with early allograft dysfunction in patients undergoing liver transplantation. Liver Transpl 2012;18:166-76.
33. Kobayashi H, Tamatani T, Tamura T, et al. The role of monocyte chemoattractant protein-1 in biliary atresia. J Pediatr Surg 2006;41:1967-72.
34. McKeating EG, Andrews PJ, Signorini DF, Mascia L. Transcranial cytokine gradients in patients requiring intensive care after acute brain injury. Br J Anaesth 1997;78:520-3.
35. Sass G, Barikbin R, Tiegs G. The multiple functions of heme oxygenase-1 in the liver. Z Gastroenterol 2012;50:34-40.
36. Tullius SG, Nieminen-Kelha M, Bachmann U, et al. Induction of heme-oxygenase-1 prevents ischemia/reperfusion injury and improves long-term graft outcome in rat renal allografts. Transplant Proc 2001;33:1286-7.
37. Tamura T, Kondo T, Ogawa K, Fukunaga K, Ohkohchi N. Protective effect of heme oxygenase-1 on hepatic ischemia-reperfusion injury through inhibition of platelet adhesion to the sinusoids. J Gastroenterol Hepatol 2012.
38. Nijboer WN, Schuurs TA, van der Hoeven JA, et al. Effects of brain death on stress and inflammatory response in the human donor kidney. Transplant Proc 2005;37:367-9.
39. Susa D, Mitchell JR, Verweij M, et al. Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. Aging Cell 2009;8:192-200.
40. Saat TC, Susa D, Roest HP, et al. A comparison of inflammatory, cytoprotective and injury gene expression profiles in kidneys from brain death and cardiac death donors. Transplantation 2014;98:15-21.

41. Junnarkar SP, Tapuria N, Mani A, et al. Attenuation of warm ischemia-reperfusion injury in the liver by bucillamine through decreased neutrophil activation and Bax/Bcl-2 modulation. J Gastroenterol Hepatol 2010;25:1891-9.
42. Straatsburg IH, Abrahamse SL, Song SW, Hartman RJ, Van Gulik TM. Evaluation of rat liver apoptotic and necrotic cell death after cold storage using UW, HTK, and Celsior. Transplantation 2002;74:458-64.
43. Karimian N, Westerkamp AC, Porte RJ. Biliary complications after orthotopic liver transplantation. Curr Opin Organ Transplant 2014;19:209-16.
44. Jay CL, Skaro AI, Ladner DP, et al. Comparative effectiveness of donation after cardiac death versus donation after brain death liver transplantation: Recognizing who can benefit. Liver Transpl 2012;18:630-40.
45. Axelrod DA, Dzebisashvili N, Lentine KL, et al. Variation in biliary complication rates following liver transplantation: implications for cost and outcome. Am J Transplant 2015;15:170-9.



Chapter 5

A signature of renal stress resistance induced by short-term dietary restriction, fasting, and protein restriction



T.C. Saat[§], F. Jongbloed[§], M. Verweij, C. Payan-Gomez, J.H.J. Hoeijmakers, S. van den Engel, C.T. van Oostrom, G. Ambagtsheer, S. Imholz, J.L.A. Pennings, H. van Steeg, J.N.M. IJzermans, M.E.T. Dollé, R.W.F. de Bruin

[§] *Authors contributed equally*

Scientific Reports

Abstract

During kidney transplantation, ischemiareperfusion injury (IRI) induces oxidative stress. Short-term preoperative 30% dietary restriction (DR) and 3-day fasting protect against renal IRI. We investigated the contribution of macronutrients to this protection on both phenotypical and transcriptional levels.

Male C57BL/6 mice were fed control food ad libitum, underwent two weeks of 30% DR, 3-day fasting, or received a protein-, carbohydrate- or fat-free diet for various periods of time. After completion of each diet, renal gene expression was investigated using microarrays. After induction of renal IRI by clamping the renal pedicles, animals were monitored seven days postoperatively for signs of IRI.

In addition to 3-day fasting and two weeks 30% DR, three days of a protein-free diet protected against renal IRI as well, whereas the other diets did not. Gene expression patterns significantly overlapped between all diets except the fat-free diet. Detailed meta-analysis showed involvement of nuclear receptor signaling via transcription factors, including FOXO3, HNF4A and HMGA1.

In conclusion, three days of protein-free diet is sufficient to induce protection against renal IRI similar to 3-day fasting and two weeks of 30% DR. The elucidated network of common protective pathways and transcription factors further improves our mechanistic insight into the increased stress resistance induced by short-term DR.

Introduction

Dietary restriction (DR) is a reduction in food intake without malnutrition[1]. Long-term DR is known to extend lifespan, increase overall health and improve resistance to multiple stressors in a wide variety of organisms[1-5]. Although the effect of DR on human lifespan is unknown, studies demonstrate a favorable impact on metabolic parameters associated with long-term health[6-8]. In addition, DR has been shown to protect against acute stressors including toxic chemotherapy[9], paracetamol intoxication[10], and oxidative stress induced by ischemia reperfusion injury (IRI)[1,11,12]. In clinical kidney transplantation, renal IRI is a major risk factor for organ damage which may result in acute kidney injury[13], primary non-function[14], delayed graft function[15], and acute and chronic rejection[16] of the graft. After kidney retrieval, cessation of the blood flow (ischemia) leads to hypoxia, nutrient deprivation, and accumulation of metabolic waste products[15,16]. Reperfusion of the ischemic kidney promotes the generation of reactive oxygen species, triggers apoptotic cell death, and promotes the activation of an inflammatory response resulting in profound tissue injury[17]. Prevention or amelioration of renal IRI could increase graft quality, and prolong graft survival. Unfortunately, no effective treatment to reduce or prevent IRI is currently available.

Using renal IRI as a model, we previously demonstrated that the benefits of DR on IRI are induced rapidly: two and four weeks of 30% preoperative DR as well as three days of fasting reduce renal injury and strongly improve survival and kidney function after renal IRI in mice[1,12]. Hence, DR is a potential intervention for living kidney donors to reduce IRI and improve the transplantation success rate. Whether the protective effect of short- and long-term DR is based on the reduction of calories per se, or specific nutrients, was first investigated in fruit flies, in which long-term protein restriction contributed more to lifespan extension than a reduction in carbohydrates[18]. In mice, glucose supplementation did not interfere with fasting-induced protection against renal IRI, which also points towards a role for specific (macro-)nutrients in inducing acute stress resistance[11].

In this study, we investigated the role of specific macronutrients in inducing resistance against renal IRI by unrestricted feeding of protein-, carbohydrate-, and fat free diets. We showed that the absence of protein for three days is sufficient to induce resistance against renal IRI and revealed common pathways and transcription factors that are implicated in the protective effect of calorie restriction, induced by two weeks of 30% DR, three days of fasting, and protein restriction.

Materials and Methods

Study design

Sample size calculation of our previous study was based on a 25% decrease of serum urea levels at time point 24 hours after renal IRI, with a standard deviation (SD) of 20% and a power of 0.8[1,11]. These experiments demonstrated that our renal IRI model was feasible and stable, and we therefore reduced the number of mice to six mice per group in the dietary intervention groups. Primary endpoint of this study was kidney function 24 hours after surgery, measured via serum urea concentrations. Secondary endpoints of this study were mortality rate in the first seven days after surgery and changes in gene expression profiles measured directly after each dietary intervention. Experimental data of two weeks 30% DR and three days of fasting groups were previously obtained. Animals were euthanized and excluded from the experiment if their body weight decreased ≥ 20% of their preoperative weight or if they developed a moribund phenotype, including ruffled fur, hunched body posture, hypothermia, and decreased activity[11,12].

Animals

C57BL/6 male mice, 10-12 weeks old (20-25 grams), were obtained from Harlan, the Netherlands. Animals were housed in individually ventilated cages (3-4 animals/cage) under standard conditions. All mice had ad libitum (AL) food and water (acidified with HCl to a pH of 2.4-2.7) except where noted. All experiments were performed with the approval of the Animal Experiments Committee of the Erasmus University Medical Center, Rotterdam, the Netherlands under the Dutch National Experiments on Animals Act and according to the ARRIVE Guidelines, Animal Research: Reporting of *In Vivo* Experiments[19].

Diets and experimental design

The control chow for the fasting and dietary restriction groups was obtained from Special Diet Services (SDS, Witham, UK). All other diets were purchased from Research Diets, Inc. (New Brunswick, NJ, USA). The macronutrient composition and energy content of all diets are shown in **Table S1**. The control diet differed from the standard chow given (Special Diet Services, SDS) in the protein source, which consisted of lactic casein protein in case of control diet, while the SDS control consisted of crude protein. These two control diets are designated as "Control" and "SDS" throughout the manuscript.

Long-term dietary intervention

Upon arrival, mice were allowed *ad libitum* (AL) access to the SDS chow for seven days. At the start of the dietary intervention period, all mice were transferred to clean cages at 4:00 pm. Mice were randomly divided into a group with 30% DR (n=5) or AL access to a carbohydrate-free (CHO-free) diet (n=6) or a fat-free diet (n=6) for 14 days, or a

protein-free diet (n=6) for 10 days (**Figure S1**). Mice in the control group for DR had AL access to the control SDS chow (n=10). The control group for CHO-free and fat-free had AL access to control type I diet (n=12). The effect of food intake was measured using pair-fed (PFed) control groups. Pair-feeding of each group was accomplished by giving the PFed groups the identical isocaloric amount of the control diet as the mice on the experimental diet had consumed the day before. The CHO-free, fat-free and protein-free diets were PFed in this manner (n=6/group). Mice with 30% DR were given 70% of the normal daily intake of mice on the control diet, which was administered once daily at 4:00 pm. Since the phenotypical effects of two weeks 30% DR were reported previously, microarray analysis was used as the only outcome for this group[1].

Short-term dietary intervention

Upon arrival, the same procedure was followed as for the long-term experiment. Mice were randomly divided into groups with AL access to control diet (n=4), a protein-free diet (n=6 per group) for three days or 30% DR for three days (n=6), or into groups with AL access to SDS chow or fasting for three days (n=5 per group). Since the phenotypical effects of three days fasting were reported previously, transcriptome analysis was the only parameter for this group[1]. For a graphical overview of the experimental setup see **Figure S1**.

Dietary intake and body weight

Food intake and body weight were measured daily. To determine calorie intake, the daily food intake was corrected for the variation in the energy content (per gram of food) in the diets as follows: food intake per mouse times the number of calories per gram of food. Change in body weight was addressed in percentages calculated by dividing the body weight measurements during the dietary intervention through body weight at onset of the intervention period times 100.

Surgical procedure

Following each dietary intervention, bilateral renal IRI was induced as previously described[1]. In brief, mice were anaesthetized via inhalation of isoflurane (5% isoflurane initially followed by 2-2.5% with oxygen for maintenance). Via midline abdominal incisions, the renal arteries and veins were exposed followed by occlusion of both renal pedicles for 37 minutes using non-traumatic vascular clamps. Purple discoloration of the kidneys confirmed ischemia macroscopically, while reperfusion was established when the color of both kidneys normalized after removal of the clamps. The incision was closed with 5/0 sutures in two layers. Following closure, mice received 0.5 ml PBS subcutaneously to compensation for fluid loss. The morning after surgery, another identical dose of PBS

was given. Mice intended for microarray analysis were sacrificed immediately after the dietary intervention, without induction of renal IRI.

Kidney function

Kidney function was determined as previously described by measuring serum urea levels in blood samples, collected via retro-orbital puncture while mice were anesthetized, before (T=0) and day 1 (T=1) after induction of renal IRI[1].

Immuno-blotting

Mouse kidney extracts from 9 different intervention diets were prepared by sonification with Soniprep 150 2-3 times 30 seconds on ice in Laemmli buffer (135 mM Tris-HCl pH 6.8, 4.5% SDS, 22.5% glycerol), supplemented with complete protease inhibitors and PhosSTOP phosphates inhibitors (Roche Diagnostics, Indianapolis, IN, USA) After sonification, lysates were centrifuged at 4 degrees Celsius for 10 minutes. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). 25µg protein was loaded on a NuPAGE 10% Bis-Tris Gel (Life Technologies LTD, Paisley, UK) and transferred to a PVDF Hybond-P (GE-Healthcare Life Sciences, Uppsala, Sweden) transfer membrane. Immunoblotting was performed with antibodies directed against S6 (#2217S Lot5; 1:1.000) and Phospho-S6 (Ser240/244; #2215 Lot14; 1:500) (Cell Signaling Technology, Danvers, MA, USA). Blotting membranes were incubated with primary antibodies overnight at 4 degrees Celsius, before they were washed and incubated with 1:5000 diluted secondary anti-rabbit-IgG-HRP antibody (GE-Healthcare Life Sciences). Detection was performed by enhanced chemiluminescence using the ECL 2 Western Blotting Substrate (Pierce Biotechnology). Levels of S6 and Phospho-S6 were semi-quantified using the ImageJ software package and S6-phospho /S6-total ratios relative to the control diet were calculated and differences between groups were assessed with a t-test (http://rsb.info.nih.gov/ij/index.html). B-Actin was used as loading control (Sigma; A5441 Lot064M4789V; 1:25.000).

Microarrays

The duration of dietary interventions that were used to study gene expression profiles was three days for all diets with exception of 30% DR, which was given for two weeks. Kidneys were obtained directly after each intervention and were snap frozen in liquid nitrogen until further analyses. An overview of the dietary interventions, the groups of mice and numbers used for phenotypical and transcriptional endpoints are summarized in **Table S2**. Total RNA was extracted using QIAzol lysis Reagent and miRNAeasy Mini Kits (QIAGEN, Hilden, Germany), following Qiagen protocol. Addition of wash buffers RPE and RWT (QIAGEN) was done mechanically by using the QIAcube (QIAGEN, Hilden, Germany) via the miRNAeasy program. Isolated RNA was and stored at -80°C. The concentration

of RNA was measured by Nanodrop (Thermo Fisher Scientific™, Breda, the Netherlands) and RNA quality was assessed using the 2100 Bio-Analyzer (Agilent Technologies, Amstelveen, the Netherlands) according to manufacturer's instructions. The RNA quality was expressed as the RNA integrity number (RIN, range 0-10). RIN values of included samples ranged between 6.6 and 8.5. Hybridization to Affymetrix HT MG-430 PM Array Plates was performed at the Microarray Department of the University of Amsterdam (the Netherlands), according to Affymetrix protocols. Four to six biological replicates were used for each group. Quality control and normalization were performed using the pipeline at the www.arrayanalysis.org website (Maastricht University, the Netherlands)[20]. Normalization was done via the Robust Multichip Average (RMA) algorithm[21]. Due to a range in hybridization dates between fasting and the other diets (i.e. September 2011 versus August 2012), normalization of the data for fasted animals and their controls was done separately. Normalization output consisted of data for 45,141 probes, with several probes corresponding to the same Gene ID. Complete raw and normalized microarray data and their MIAME compliant metadata have been deposited at the Gene expression Omnibus (GEO) database GEO GSE65656 (www.ncbi.nlm.nih.gov/geo). After normalization, outliers were found in the control SDS (control 30% DR), the 3-day fasting and 3-day fat-free diet groups, defined as a deviation in array-array intensity correlations, principal component analysis and cluster dendograms. These outliers were excluded from further analyses (**Table S2**).

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS (version 21.0) and GraphPad Prism (version 5.0). Differences in serum urea concentrations were compared by Mann-Whitney U tests, food intake via the paired t-test and survival rates were analyzed by Log-rank tests. A p-value of <0.05 was considered significant. Microarray analyses were performed using the free software package R (R foundation). Gene expression data were compared using the Linear Models for Microarray Data (limma) method with correction for multiple testing using the false discovery rate (FDR) according to Benjamini and Hochberg[22]. Fold changes were expressed as the geometric mean per diet group against the corresponding ad libitum fed control group. Cutoff values for a significant difference were put at FDR <5% with fold change ≥1.5. The enrichment factor (EF) was calculated via the formula: $EF=nAB/((nA\times nB)/nC)$, where nA is the number of differentially expressed probe sets (DEPS) in experimental group A, nB the number of DEPS in experimental group B, nC the number of total genes in the microarray, and nAB the number of common DEPS between nA and nB. Data integration of microarrays hybridized in different dates was performed with meta-analysis. The methodology applied was combining rank orders. It is a non-parametric approach based on rank orders. The R package RankProd implemented in

INMEX was used. In summary, for each dataset, the fold changes (FC) were calculated for all possible pairwise comparisons. The ranks of the fold changes within each comparison were used to calculate the rank product for each gene. To assess the null distribution of the rank product within each data set, a permutation test was performed. The process was repeated several times to compute the P-value and false discovery rate (FDR) associated with each gene. A gene was selected as differentially expressed if it had an FDR <5% and an absolute combined FC ≥1.5. Functional annotation and analyses were performed using Ingenuity software (http://www.ingenuity.com/products/ipa). The prediction inhibition or activation of the upstream transcription regulators is calculated via de statistical z-score based on the observed gene expression changes in our dataset. Calculating the z-score reduces the chance of significant predictions based on random data (http://ingenuity.force.com/ipa/articles/Feature_Description/Upstream-Regulator-Analysis). Cutoff values for a significant activation or inhibition were met with a z-score of ≥ 2 or ≤-2, respectively.

Results

Absence of dietary protein induces protection against renal IRI

To determine the effect of short-term macronutrient deficiency on renal IRI, we provided 10-day, 14-day or 3-day regimens of protein-free, carbohydrate (CHO)-free and fat-free diets before inducing renal IRI. As previously shown, mice that were fed a protein-free diet for six or 14 days tend to voluntarily decrease their food intake as compared to animals that were fed a normal diet[23]. We indeed found that mice fed a protein-free diet for 10 days decreased their dietary food intake by approximately 30% (**Figure S2A**), resulting in significant body weight loss up until 20% on day 10 (**Figure S2B**). The reduction in food intake and body weight was less substantial in mice fed a CHO-free diet for 14 days. A fat-free diet for 14 days led to a small increase in body weight. Only the protein-free diet improved survival (**Figure S2C**) and kidney function (**Figure S2D**) following renal IRI. However, due to the reduction in food intake and body weight, the effect of the absence of protein per se could not be disentangled from the effect of calorie restriction.

Subsequently, to separate the effect of the absence of protein from calorie restriction, mice were fed a protein-free diet for three consecutive days. We first showed that survival and kidney function of mice receiving 30% DR for three days did not differ from mice fed ad libitum (**Figure 1A, B**). Mice fed the protein-free diet for three days had significantly improved survival (**Figure 1C**) and kidney function compared to control mice ($P<0.05$) (**Figure 1D**). The energy intake during the 3-day protein-free dietary intervention was decreased, but did not significantly differ from the intake of animals fed the control

diet for three days (P=0.13) (**Figure 1E**). Body weight of mice on the protein free diet decreased by 9%, while body weight of mice that were fed a control diet did not change. Mice receiving 30% DR for three days lost about 8% of their body weight (**Figure 1F**).

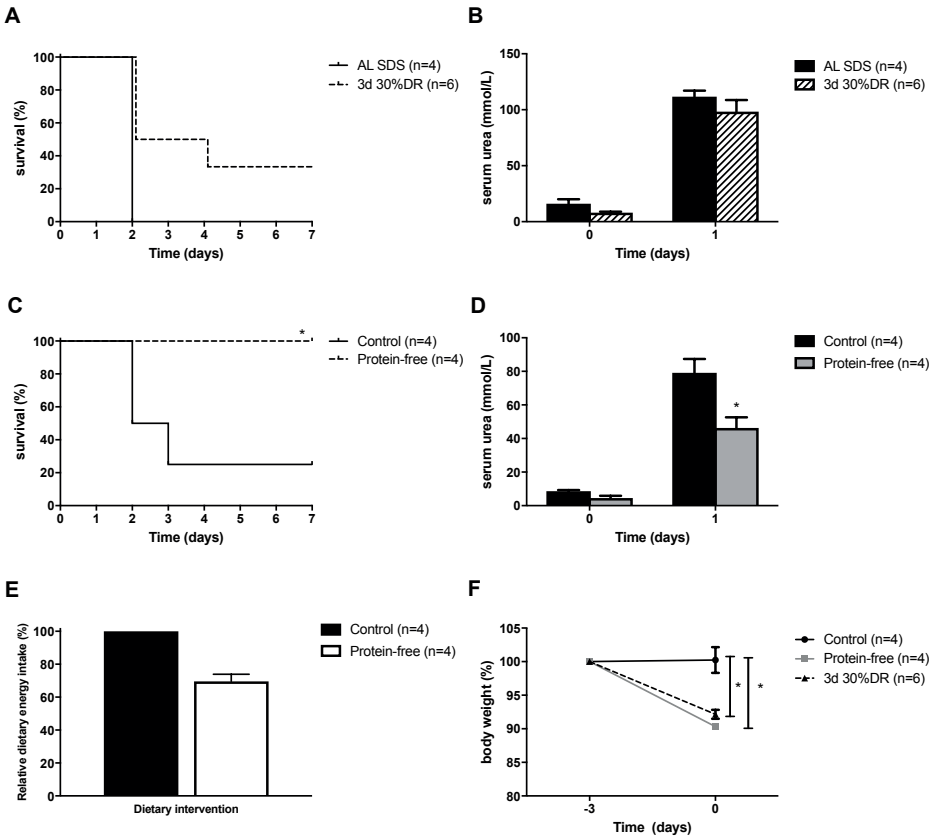


Figure 1. A protein-free diet protects against renal IRI. **(A)** Three days of 30% DR did not protect against renal IRI. **(B)** Serum urea levels before and one day after renal IRI did not differ between mice fed ad libitum (AL) or three days of 30% DR. **(C)** Three days of protein-free diet improved survival compared with AL fed controls. **(D)** Serum urea levels of AL fed protein-free mice were significantly lower compared to AL fed control mice (P<0.05). **(E)** Three days of protein-free diet resulted in 30% calorie restriction. **(F)** Mice fed a protein-free diet or a 30% DR diet for three days lost significantly more body weight than mice fed the control diet. Body weight changes did not differ between the protein-free diet and 3-day 30% DR. AL Control = ad libitum fed SDS chow, Control = control diet for the macronutrient diets, protein free = ad libitum fed protein-free diet. *P <0.05.

Common gene expression profiles between macronutrient-free diets, fasting and DR

To examine, in an unbiased manner, the transcriptomic response of the kidney, microarray analysis was performed on 45,141 probe sets in kidney samples after three days of

fasting, three days and two weeks of 30% DR, three days of protein-, fat- or CHO-free diet, each compared to its corresponding AL fed control group. As the three days of fasting on the one hand and two weeks 30% DR and 3-day 30% DR on the other hand were each performed as individual cohorts using their own control group, we had three different control groups for all interventions combined. No significant differences in gene expression profiles were observed between the different control diets (data not shown). The highest number of significantly differentially expressed probe sets (DEPS) was found after three days of fasting, namely 2,604, of which 1,268 were up- and 1,336 down-regulated. Two weeks of 30% DR gave rise to a five times lower number: 492 DEPS, of which 265 were up-regulated and 227 were down-regulated. Three days of protein-free diet induced 391 DEPS, with 230 probe sets up- and 161 down-regulated. Of the non-protective diets, the 3-day fat-free diet did not induce any DEPS when compared to control diet fed mice, while three days of a CHO-free diet induced 1,717 DEPS containing 613 up- and 1,104 down-regulated sets. Three days of 30% DR resulted in 454 DEPS, of which 284 up- and 170 down-regulated.

Several analytic approaches were performed to compare the transcriptomic response between all diets. First, a comparison was made based on the number of overlapping DEPS and the corresponding significance of the overlap (**Table 1**). Based on the maximum relative overlap, the 3-day protein-free diet and 3-day fasting demonstrated the most resemblance to each other with 222 DEPS in common, corresponding to 53.3% relative overlap. Two-weeks 30% DR showed 45.9% relative overlap with three days of fasting, with 247 DEPS in common. To compare the significance of these overlapping fractions, the enrichment factors (EF) with corresponding p-values were calculated. Comparison of the two identically modified diets differing only in intervention time, namely 3-days 30% DR and two weeks 30% DR, resulted in the highest EF with a 39 times higher number of DEPS in common than would have been expected by chance (**Table 1**). Comparison of the 3-day protein-free diet with 3-day 30% DR resulted in an EF of 29. All enrichments were significant, with values lower than 8.66E-48, suggesting a significant common transcriptomic response between dietary interventions with the exception of a 3-day fat-free diet. As directionality of the DEPS was not accounted for in this comparison, we explored dendographic heat maps. Since the 3-day fasting dataset was hybridized on a different date, the date of hybridization caused a stronger effect than the biological signal (**Figure S3**). It was therefore not possible to integrate all complete datasets in one informative heat map analysis. As a solution, we assumed three days of fasting to represent the widest transcriptomic protective response, and all data sets were limited to its highest number of 2,604 DEPS. The resulting heat map (**Figure 2A**) shows that the majority of the DEPS have similar directionalities, with the lowest expression levels in the fat-free diet. The horizontal dendogram, based on the (dis)similarity between expression data for probe sets, shows that three days of fasting

Table 1. Comparison of the overlapping DEPS between the five dietary interventions and their corresponding P-value and enrichment factor

Comparison	Overlapping DEPS	Relative overlap (%)*	Enrichment factor	*P*-value
Protein-free vs. Fasting	222	53.3	9.8	5.27E-169
2wks 30% DR vs. Fasting	247	45.9	8.7	8.88E-171
3d 30% DR vs. Fasting	208	42.0	8.7	3.12E-156
2wks 30% DR vs. 3d 30% DR	195	28.1	39.4	3.44E-270
Protein-free vs. CHO-free	113	24.9	6.0	5.84E-55
Fasting vs. CHO-free	584	23.7	5.9	3.73E-300
2wks 30% DR vs. CHO-free	122	20.4	8.2	5.71E-76
3d 30% DR vs. CHO-free	107	19.6	5.8	8.66E-48
Protein-free vs. 3d 30% DR	116	18.5	29.5	2.22E-138
Protein-free vs. 2wks 30% DR	95	15.2	22.3	1.15E-99

The highest percentage of relative overlap was found between three days of protein-free diet and two weeks of 30% DR. The enrichment factor indicates the number of times the overlapping DEPS is higher than expected by chance. All diets showed a significantly overlapping number of DEPS, as shown by the corresponding p-values in the last column. *Relative overlap is calculated by the number of DEPS in common between the two groups, divided by the total number of unique DEPS across both groups, relative to the theoretical maximum overlap according to this formula.

portrays the largest differences with the other groups. However, this was an assumption, since the heat map is based on the probe sets differentially regulated after three days of fasting. Compared to three days of fasting, a 3-day CHO-free diet is the least clustered with the other dietary groups. The 3-day protein-free diet clusters closely with the non-protective 3-day fat-free diet, based on the number of probe sets as well as the expression levels. Three days 30% DR had a similar cluster pattern as two weeks of 30% DR. Subsequently, the same set of 2,604 DEPS significantly regulated after fasting compared to AL fed controls, was used for a principal component analysis (PCA) among the macronutrient free diets, two weeks-, and three days 30% DR and their control diets (**Figure 2B**).

Samples from the control diets and the 3-day fat-free diet cluster together, with high overlap and corresponding directionality between the groups. With a large distance on the principal component (PC) 2 axis, a similar clustering was seen among the 3-day 30% DR and two weeks 30% DR samples. The protein-free diet had its own cluster, separated on both PC axes from the other groups. The 3-day CHO-free diet resembled the DR diets, but showed no overlap with these diets and showed a large dissimilarity to the other groups. To compare the transcriptomic responses between all dietary interventions, all DEPS were visualized in a Venn diagram (**Figure 3A**). This revealed a total of 40 overlapping DEPS in the three protective diets, while 30 overlapping DEPS were also present in the non-protective CHO-free diet. The genes corresponding to the 70 DEPS in common

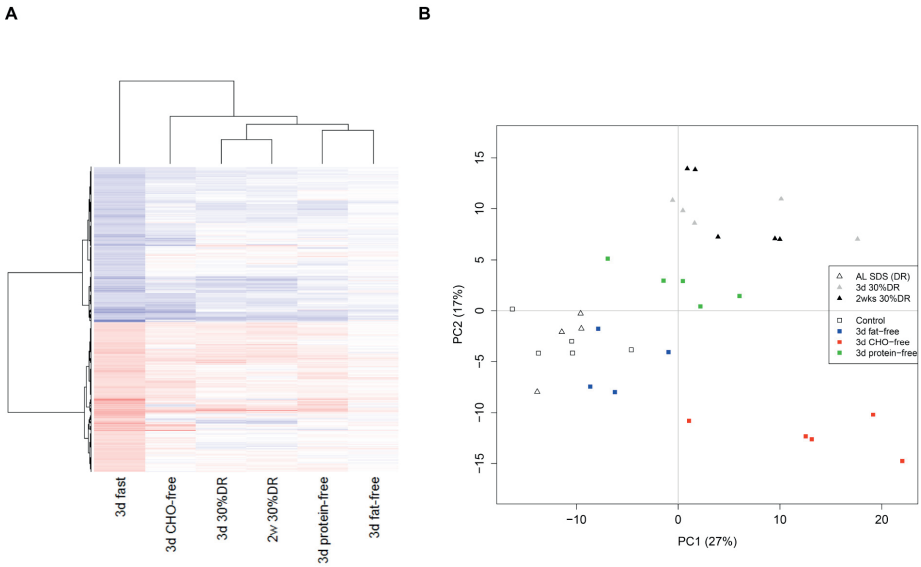


Figure 2. Heat map and PCA plots of directional and cluster patterns in all dietary interventions based on the differentially expressed probe sets (DEPS) after three days of fasting compared to its control group. **(A)** The majority of the DEPS in the kidney after three days of fasting showed the same directionality in the other dietary interventions. The 3-day CHO-free diet was the least clustered with the other diets, followed by two weeks 30% DR and a 3-day protein-free diet. The fat-free diet, showing no significant DEPS, clustered together with a 3-day protein-free diet. Red= up-regulation, blue = down-regulation, white = no change. CHO-free = carbohydrate-free. **(B)** Principal component analysis (PCA) plot, based on the 2604 significantly regulated probe sets after three days fasting compared to the control diet fed animals. Both two weeks 30% DR and three days of 30% DR diet clustered close to each other based on the DEPS found after three days of fasting. The two control diets, control and SDS diet, clustered together with the non-protective fat-free diet. Three days of CHO-free diet positioned closely to two weeks and three days of 30% DR, but did not overlap with these diets. The protein-free diet had its own cluster, separated from the other groups on both PC axes.

are listed in **Table S3**. Comparing 3-day 30% DR with the protective diets, a similar pattern was observed; only 15 DEPS overlapped in the three protective diets, while 47 DEPS were also present in the non-protective 3-day 30% DR diet (**Figure 3B**). However, both numbers of overlapping genes appeared too small to perform pathway analysis with the aim to find a common denominator of protection against renal IRI, and therefore an alternative approximation for analysis was used.

Pathway analyses and transcription factor analyses

To explore the biological value of these transcriptomic responses, we used an individual approximation to identify significantly enriched pathways. The highest enriched pathways after the different dietary interventions were ranked by their –log p-value and summarized in **Table S4**. No clear pattern in overlapping pathways between all diets, or between the protective diets and the non-protective CHO-free diet, was observed. One

pathway that emerged was the NRF2-mediated oxidative stress response pathway, since this pathway was activated by four out of five dietary interventions (**Table S4**).

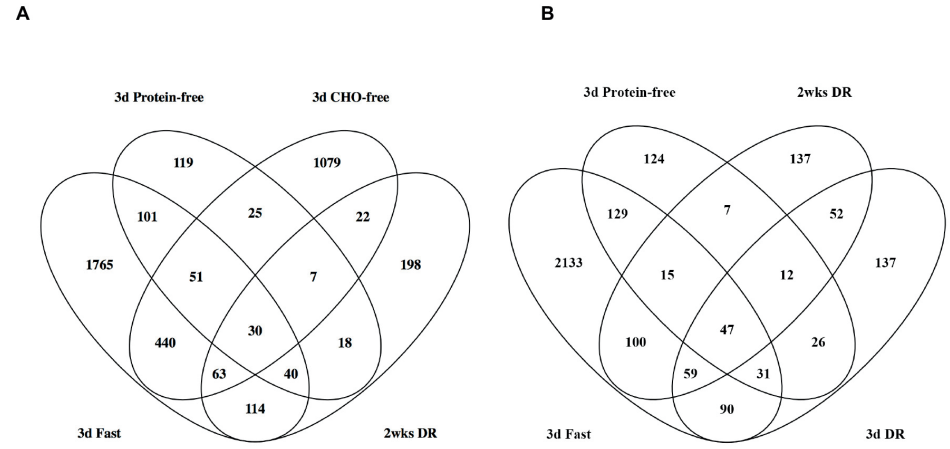


Figure 3. Venn diagram of multiple dietary interventions combined. **(A)** Venn diagram showing the number of DEPS after three days of fasting, two weeks 30% DR, three days of protein-free diet, three days of CHO-free diet, and their overlap with each diet. Thirty DEPS are differentially regulated in all four diets including the non-protective CHO-free (centre). The protective diets have 70 DEPS in common, of which 40 of those in common with the CHO-free diet are excluded (lower right). **(B)** Venn diagram showing the number of DEPS after three days of fasting, three days of a protein-free diet, two weeks and three days of 30% DR with their overlap. Forty-seven DEPS are differentially regulated in all fours diets (centre). A total of 15 DEPS were overlapping between the three protective diets.

To further identify a common protective response and dissect it from the response of the non-protective CHO-free diet, a more comprehensive meta-analytic approximation was used. A combining rank orders methodology was implemented to prevent bias of the results based on outliers as well as the stronger transcriptomic response after three days of fasting[24]. By eliminating all significant probe sets induced by the CHO-free diet in this meta-analysis, only 140 DEPS remained. Pathway analysis revealed no significant pathways (data not shown). Theorizing that the protective response might be partially overlapping with the response of the non-protective CHO-free diet, we repeated the meta-analysis with the three protective diets and the CHO-free diet into the approximation, thus not excluding the 3-day CHO-free diet. This meta-analysis yielded 640 DEPS. The majority of these DEPS in common showed a similar fold change and directionality among the three protective dietary interventions.

A pathway analysis of these DEPS demonstrated regulation of nuclear receptor signaling as well as inhibition of cellular stress and injury and biosynthesis pathways amongst the top 10 overrepresented pathway categories (**Table 2**). Adding the 3-day 30% DR diet in the meta-analysis yielded 279 DEPS. No significant enriched pathways emerged. The 10 highest enriched pathways are depicted in **Table S5**. To further explore the regulated

Table 2. Overview of the top 10 overrepresented canonical pathways in the meta-analysis ranked by their –log *P*-value.

Meta-analysis				
Canonical Pathway	**Pathway Category**	**P-value**	**Genes Ratio**	**Z-score**
LXR/RXR Activation	Nuclear Receptor Signaling	7.24E-09	16/121 (13.2%)	+0.302
FXR/RXR Activation	Nuclear Receptor Signaling	3.63E-06	13/127 (10.2%)	N/A
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	2.24E-05	16/219 (7.3%)	-2.646
Superpathway of Cholesterol Biosynthesis	Fatty Acids and Lipids Biosynthesis, Sterol Biosynthesis	2.40E-05	6/28 (21.4%)	N/A
NRF2-mediated Oxidative Stress Response	Cellular Stress and Injury	3.72E-05	14/180 (7.8%)	-1.000
Aryl Hydrocarbon Receptor Signaling	Cell Cycle Regulation; Apoptosis; Xenobiotic Metabolism, Nuclear Receptor Signaling	5.13E-05	12/140 (8.6%)	+1.633
PXR/RXR Activation	Nuclear Receptor Signaling	9.12E-05	8/67 (11.9%)	N/A
Intrinsic Prothrombin Activation Pathway	Cardiovascular Signaling; Cellular Stress and Injury	3.47E-03	5/29 (17.2%)	N/A
Superpathway of Geranylgeranyldiphosphate Biosynthesis I	Cofactors, Prosthetic Groups and Electron Carriers Biosynthesis	3.98E-03	4/17 (23.5%)	N/A
Aldosterone Signaling in Epithelial Cells	Cardiovascular Signaling; Nuclear Receptor Signaling	4.57E-03	11/152 (7.2%)	N/A

The top 10 overrepresented pathways derived from the 640 DEPS in common between three days of fasting, two weeks 30% DR, three days of a protein-free diet and three days of a carbohydrate-free diet. These pathways are mostly involved in regulation of nuclear receptor signalling (five out of 10), biosynthesis signalling (two out of 10) and cellular stress and injury (two out of 10).

DEPS and pathways in relation to the protective response against renal IRI, we examined the involvement of upstream transcription factors (TFs) in the DEPS that emerged from the meta-analysis. In the meta-analysis, 16 TFs were identified of which 12 were predicted to be activated and six inhibited (**Table 3**). Critical denominators might be TFs showing the same directionality in the protective diets, but are oppositely directed or not regulated in the non-protective CHO-free diet. The TFs complying with these criteria, in descending order of absolute z-score, were forkhead box O3 (FOXO3), heat shock factor protein 1 (HSF1), and high mobility group AT-hook 1 (HMGA1).

Furthermore, hepatocyte nuclear factor 4-alpha (HNF4α) was highly activated in the protective diets, but only minimally in the non-protective CHO-free diet. Also, sterol regulatory element-binding transcription factor 1 (SREBF1) and 2 (SREBF2) were significantly down-regulated after three days of fasting and in the protein-free diet, activated in the CHO-free diet but not regulated after two weeks 30% DR. The non-protective 3-day 30% DR diet showed similar results as the protective diets, since all TFs after three

Table 3. List of upstream transcription factors after meta-analysis with corresponding z-scores in the different dietary interventions.

Dietary intervention / Transcription Factor	Meta-analysis	3-day fasting	2 weeks 30% DR	Protein-free	CHO-free	3-day 30%DR
SMAD7 – SMAD family member 7	+2.890	+0.718	-0.128	+2.466	N/A	N/A
* **FOXO3 - Forkhead box O3**	+2.729	+3.303	+1.042	+1.701	-0.314	+1.893
FOXO1 - Forkhead box O1	+2.692	+1.599	-0.109	+0.269	-1.850	+0.360
* **HNF4α – Hepatocyte nuclear factor 4 alpha**	+2.462	+2.122	+1.179	+2.089	+0.109	+1.327
MYCN – v-myc avian myelocytomatosis viral oncogene	+2.414	+1.982	N/A	+1.245	-2.464	+1.091
CLOCK – Circadian Locomotor output Cycles Kaput	+2.373	+1.980	+1.119	N/A	+1.940	+1.925
*** HMGA1 – High mobility group AT-hook 1**	+2.051	+1.432	+2.177	+0.356	N/A	+0.785
MED1 – Mediator complex subunit 1	+2.008	+2.058	N/A	N/A	+0.102	N/A
SPDEF – SAM pointed domain containing ETS transcription factor	+2.000	+2.170	N/A	+1.000	N/A	N/A
LYL1 – Lymphoblastic leukemia associated hematopoiesis regulator 1	+2.000	+2.000	N/A	N/A	N/A	+1.982
SIM1 – Single-minded homolog 1	+2.000	+0.557	N/A	N/A	N/A	N/A
ARNT2 – Aryl hydrocarbon receptor nuclear translocator 2	+2.000	+0.557	N/A	N/A	N/A	N/A
GLI1 – GLI family zinc finger 1	-2.183	N/A	N/A	+0.057	N/A	N/A
*** HSF1 – Heat shock factor protein 1**	-2.697	-1.659	-2.653	-2.376	+0.462	-0.451
SREBF2 - Sterol regulatory element-binding transcription factor 2	-2.923	-3.420	N/A	-0.243	+0.660	N/A
SREBF1 - Sterol regulatory element-binding transcription factor 1	-3.889	-2.794	N/A	-1.304	+1.229	-0.779

Upstream regulator analysis of the DEPS found in common after three days of fasting, two weeks 30% DR and three days of protein-free diet (meta-analysis) revealed 16 transcription factors (TFs) significantly regulated, of which 12 were activated and four were inhibited. Highest activated TFs were SMAD7 and FOXO3. *TFs of interest that are not or oppositely regulated in the non-protective CHO-free diet.

days of 30% DR were similarly regulated as after three days of fasting, two weeks 30% DR and the three days of protein-free diet.

The validity of these findings was further examined by determining mRNA expression levels. The expression levels of FOXO1 were significantly higher after all diets except the fat-free diet (**Figure S4A**), while FOXO3 was significantly up-regulated in all diets except after two weeks of 30% DR (**Figure S4B**). FOXO4 was only significantly up-regulated after three days of protein-free and three days of CHO-free diet (**Figure S4C**). HNF4α was not significantly regulated after any of the dietary interventions (**Figure S4D**). The mRNA expression level of down-regulated transcription factor SREBF1 was only significantly down-regulated after three days of fasting (**Figure S4E**), while SREBF2 was significantly down-regulated after three days of fasting and three days of protein-free diet (**Figure S4F**).

Target pathways possibly involved in the protective effect of renal IRI

Various pathways have been proposed to be involved in the protective response against renal IRI induced by DR and protein restriction. One of these is the eukaryotic translation factor 2 (eIF2α) signaling pathway, in which eIF2α is phosphorylated by the general control nonderepressible 2 (*Gcn2*) kinase, thereby inhibiting initiation of translation[23]. The role of *Gcn2* and the eIF2α pathway is subject of debate, and the relevance of this pathway still needs to be elucidated[23,25]. Our microarray analyses showed a significant up-regulation of eIF2α transcription factor only after three days of fasting, while the *GCN2* gene and other target genes of the eIF2α pathway were not significantly differentially regulated after any of the dietary interventions. The mammalian Target of Rapamycin (mTOR) signaling pathway mediates between growth factors, hormones and nutrients to regulate essential cellular functions including survival and protein translation. Inhibition of the mTOR pathway has been demonstrated to increase lifespan in various animal species[26,27] mTOR is part of mTOR complex 1 (mTORC1), a nutrient sensor complex that is involved in induction of oxidative stress resistance[28]. We found a down-regulation of mTOR after two weeks 30% DR (-0.6; P<0.01) and three days of a protein-free diet (-1.7; P<0.001), while MTORC1 (-1.6; P<0.01) and *mTOR* (-0.6; P<0.001) were down-regulated after three days of fasting. Both non-protective diets 3-day CHO-free (+0.8; P<0.01) and 3-day 30% DR (+1.3; P<0.001) showed an up-regulation of mTOR. mTOR activity was examined at protein level by examining ribosomal protein S6 phosphorylation through immunoblotting in kidney extracts from all intervention and control groups. S6 is a downstream target of mTOR through S6 kinase[29]. Compared to the corresponding control group, three days of fasting showed a significant two-fold increase in relative ribosomal protein S6 phosphorylation (**Figure 4**). In the other dietary interventions, a large variation in phosphorylation levels was observed which did not reach statistical significance.

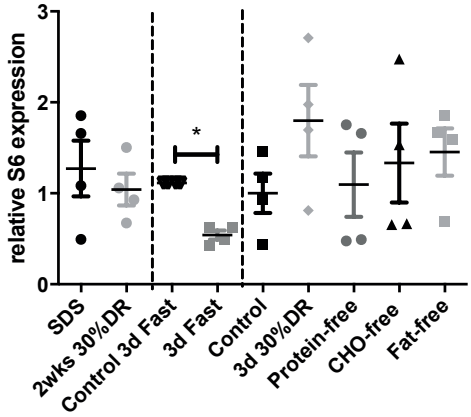


Figure 4. Relative S6 phosphorylation signals in kidney after various diet interventions. The ratio of phosphorylated S6 over total S6 was significantly lower after three days of fasting compared to its control diet. All other dietary interventions showed a large variation in phosphorylation levels that did not reach statistical significance.

Discussion

Since the discovery of the beneficial effects of short-term dietary restriction (DR) on stress resistance, optimizing its duration and content to eventually lead to a clinical applicable DR regimen has been an important part of the body of literature about DR[2,11,23]. Previously we have shown that two and four weeks of 30% DR as well as three days of fasting decreased morbidity and mortality, and improved kidney function in a murine renal IRI model[1,12]. In the present study, we show that a protein-free diet administered for only three days is sufficient to induce similar protection, whereas fat- and carbohydrate-free diets did not. Initially, we attempted longer periods of diet interventions, but found that mice fed a protein-free diet during 10 days applied self-restriction of approximately 30% of their normal intake (**Figure S2**). Therefore, the possible beneficial effects of calorie and protein restriction on kidney function and survival were indistinguishable. In a recent publication, Peng et al.[23] found that mice fed a protein-free diet for six days restricted calorie intake. However, they stated that corrected for body weight, their calorie intake was similar to that of ad libitum fed mice. It is unknown whether six days of 30% DR induces protection against IRI, therefore a distinction between protein restriction and calorie restriction is still difficult to make. We showed that three days of 30% DR does not induce protection against renal IRI, and therefore we could disentangle the effects of calorie and protein restriction per se (**Figure 1**). A protein-free diet, given for three days induced protection whereas both a CHO-free and fat-free diet given for three or 14 days did not protect against renal IRI (**Figure S2**). A recent publication by Solon-Biet et al.[20] showed that the ratio of proteins and carbohydrates rather than DR per se influenced the lifespan of mice as well as metabolic parameters such as insulin and lipids. Since proteins fully substituted the carbohydrates in our CHO-free diet, and carbohydrates fully substituted the proteins in our protein-free diet, these diets further emphasize the importance of the ratio between proteins and carbohydrates (**Table S1**).

Although the beneficial effects of both long-term and short-term DR have been acknowledged, the mechanisms underlying DR are still subject of investigation. Various pathways, factors and genes have been proposed to play a central role in the protective effects[30], but attempts to validate these yielded conflicting results[31-34]. We produced extensive expression datasets of diets proven to be either protective or not protective against renal IRI. Gene expression profiles of the non-protective CHO-free diet showed a considerable overlap with gene expression profiles of protective diets, namely three days of fasting, two weeks 30% DR and three days of protein-free diet (**Figure 3**). A PCA plot demonstrated the partial different directionality of gene expression in the CHO-free diet compared to the other dietary interventions. The 3-day 30% DR diet also showed considerable overlap with the protective diets, mainly with two weeks 30% DR, and a partially different but also partially overlapping directionality in response. These find-

ings could either indicate that overlapping probe sets and pathways are not involved in the induction of protection against renal IRI, or that they may require additional changes in other probe sets to induce this effect. Particularly, the striking similarity between three days and two weeks 30% DR suggests that three days is sufficient to activate a transcriptomic response which is not yet sufficient to induce phenotypical protection. In addition, the non-protective fat-free diet showed strong similarity with the protective 3-day protein-free diet, but with gene expression levels that were lower. These results indicate that the directionality, the number of probe sets and the expression levels are of importance in order to induce protection against renal IRI. To provide structure in the infinite amount of data generated by microarray analysis, we used meta-analytic approximations to specify for overlapping pathways and factors in all diets. A specific meta-analysis in which the DEPS were oppositely regulated in the protective diets versus the non-protective CHO-free diet, resulted in only 160 DEPS and yielded no additional pathways of interest compared to a meta-analysis including the CHO-free diet. In this meta-analysis several TFs, including FOXO3 and HNF4α, remained significantly up-regulated in the protective diets whilst not or oppositely regulated in the CHO-free diet (data not shown).

Activation of nuclear receptor signaling pathways dominated the top 10 overrepresented pathways after three days of fasting, two weeks of 30% DR and three days of protein-free diet (**Table 2**). Specific retinoid receptors, including the retinoid X receptor (RXR), the pregnane X receptor (PXR) and the peroxisome proliferator-activated receptor (PPAR)[35], were also prominently up-regulated in our analysis, pointing towards a pivotal role for nuclear receptor signaling.

Nuclear receptors are transcription factors that can be activated by steroid hormones and lipid-soluble agents, such as the retinoid acids (RAs)[36]. Administration of RAs induces many of the beneficial effects observed after DR. DR is able to ameliorate age-related insulin resistance and degenerative brain diseases, and similar results have been described after treatment with RAs[37-39]. Both DR and administration of RAs are able to protect from ischemic stroke in the brain[40-42]. Signaling pathways activated by the interaction between RAs and nuclear receptors have also been considered to have tumor-, and immune suppressive effects, just as DR[9,36,43].

Involvement of nuclear receptor signaling is further supported by the upstream TF analysis (**Table 3**), since the majority of the activated or inhibited TF could be directly or indirectly linked to the activation of nuclear receptors. The activated TFs in all protective diets that were oppositely or not regulated in the non-protective CHO-free diet are FOXO3, HNF4α, HMGA1 and HSF1. A role for each of these four TFs in increased stress resistance has been previously observed. For example, FOXO3 phosphorylation via the c-Jun N-terminal kinase -pathway results in its nuclear inclusion and activation of various processes involved in cellular stress resistance, biosynthesis, cell cycle regulation as

well as apoptosis and autophagy[44,45]. A fasting-induced interaction, mediated by insulin signaling, with members of the FOXO family and RXR has been described as well[46]. HMGA1 is a downstream target of the insulin receptor pathway and could function as an important nuclear factor mediating the binding of FOXO proteins to other nuclear receptors, including the retinoid nuclear receptor family and thereby regulating insulin target genes[47].

HNF4α is a nuclear TF that is involved in the development as well as in the metabolism of mainly the liver and kidney[48]. Up-regulation of HNF4α occurs via co-stimulation of LXR and FXR and usually depends on the presence of low levels of stressors, such as interleukin-1 and tumor necrosis factor alpha. Transcriptional activity of HNF4α is also regulated indirectly by insulin through the action of FOXO1[48]. Activation of HNF4α results in inhibition of the activity of metabolic sensors, including SREBF and the mammalian target of Rapamycin (mTOR)[49,50]. This leads to down-regulation of metabolism, in particular cholesterol metabolism, and may contribute to increased stress resistance via the down-regulation of mTOR[50,51]. We showed that the activity of mTOR, as determined by the phosphorylation of the ribosomal protein S6, was significantly down-regulated after three days of fasting. A trend towards lower phosphorylation levels was seen after the other two protective diets: two weeks 30% DR and a 3-day protein-free diet. These data indicate that mTOR may play an important role in the protection against IRI, which may vary according to the type of nutrient deprivation that is offered.

Another pathway involved in nutrient sensing is GCN2, of which eIF2α is an important downstream target. GCN2 becomes transcriptionally activated by deprivation of amino acids and phosphorylates eIF2α, which leads to the activation of pathways involved in stress resistance. We did not find transcriptional regulation of the GCN2 gene in any of the three protective diets. Although our data do not preclude posttranscriptional regulation of this pathway, they corroborate with those of Robertson et al.[52], who observed that GCN2 signaling was not required for protection against renal IRI by protein restriction.

In summary, we demonstrated that three days of a protein-free diet in mice protects against renal IRI, similar to two weeks of 30% DR and three days of fasting. Comparative transcriptional analysis of kidney tissue following these dietary interventions demonstrated a significant overlap in differentially expressed genes and pathways, which are involved in resistance against oxidative damage induced by renal IRI. A meta-analysis of pathways and TFs indicates that DR up-regulates at least four TFs that activate a transcriptional response, which, in turn, increases nuclear receptor signaling dependent and independent cellular stress resistance. However, our attempt to understand the beneficial effects of different dietary restriction regimens on renal IRI by transcriptome analysis suggests that pivotal molecular changes also occur beyond the transcriptional level, and that additional 'omics" analyses, including proteomics are needed to come to

a complete understanding. Therefore, more research is warranted to further elucidate the role of these pathways in the induction of acute stress resistance by short-term DR, which may ultimately lead to "dietary restriction mimetic" therapeutic strategies that exploit the benefits of dietary restriction in humans.

References

1 Mitchell, J. R. *et al.* Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell* **9**, 40-53, doi:10.1111/j.1474-9726.2009.00532.x (2010).

2 Fontana, L., Partridge, L. & Longo, V. D. Extending healthy life span--from yeast to humans. *Science* **328**, 321-326, doi:10.1126/science.1172539 (2010).

3 Robertson, L. T. & Mitchell, J. R. Benefits of short-term dietary restriction in mammals. *Exp Gerontol* **48**, 1043-1048, doi:10.1016/j.exger.2013.01.009 (2013).

4 Raffaghello, L. *et al.* Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci U S A* **105**, 8215-8220, doi:10.1073/pnas.0708100105 (2008).

5 Vigne, P., Tauc, M. & Frelin, C. Strong dietary restrictions protect Drosophila against anoxia/reoxygenation injuries. *PLoS One* **4**, e5422, doi:10.1371/journal.pone.0005422 (2009).

6 Heilbronn, L. K. *et al.* Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. *JAMA* **295**, 1539-1548, doi:10.1001/jama.295.13.1539 (2006).

7 Weiss, E. P. *et al.* Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. *Am J Clin Nutr* **84**, 1033-1042 (2006).

8 Fontana, L. & Klein, S. Aging, adiposity, and calorie restriction. *JAMA* **297**, 986-994, doi:10.1001/jama.297.9.986 (2007).

9 Huisman, S. A., Bijman-Lagcher, W., JN, I. J., Smits, R. & de Bruin, R. W. Fasting protects against the side effects of irinotecan but preserves its anti-tumor effect in Apc15lox mutant mice. *Cell Cycle* **14**, 2333-2339, doi:10.1080/15384101.2015.1044170 (2015).

10 Antoine, D. J., Williams, D. P., Kipar, A., Laverty, H. & Park, B. K. Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. *Mol Med* **16**, 479-490, doi:10.2119/molmed.2010.00126 (2010).

11 Verweij, M. *et al.* Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. *Transplantation* **92**, 752-758, doi:10.1097/TP.0b013e31822c6ed7 (2011).

12 Jongbloed, F. *et al.* Preoperative fasting protects against renal ischemia-reperfusion injury in aged and overweight mice. *PLoS One* **9**, e100853, doi:10.1371/journal.pone.0100853 (2014).

13 Bonventre, J. V. & Yang, L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest* **121**, 4210-4221, doi:10.1172/JCI45161 (2011).

14 Brook, N. R., Waller, J. R. & Nicholson, M. L. Nonheart-beating kidney donation: current practice and future developments. *Kidney Int* **63**, 1516-1529, doi:10.1046/j.1523-1755.2003.00854.x (2003).

15 Perico, N., Cattaneo, D., Sayegh, M. H. & Remuzzi, G. Delayed graft function in kidney transplantation. *Lancet* **364**, 1814-1827, doi:10.1016/S0140-6736(04)17406-0 (2004).

16 Kosieradzki, M. & Rowinski, W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant Proc* **40**, 3279-3288, doi:10.1016/j.transproceed.2008.10.004 (2008).

17 Snoeijs, M. G., van Heurn, L. W. & Buurman, W. A. Biological modulation of renal ischemia-reperfusion injury. *Curr Opin Organ Transplant* **15**, 190-199, doi:10.1097/MOT.0b013e32833593eb (2010).

18 Mair, W., Piper, M. D. & Partridge, L. Calories do not explain extension of life span by dietary restriction in Drosophila. *PLoS Biol* **3**, e223, doi:10.1371/journal.pbio.0030223 (2005).

19 Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother* **1**, 94-99, doi:10.4103/0976-500X.72351 (2010).

20 Solon-Biet, S. M. *et al.* The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metab* **19**, 418-430, doi:10.1016/j.cmet.2014.02.009 (2014).

21 He, X. *et al.* PIK3IP1, a negative regulator of PI3K, suppresses the development of hepatocellular carcinoma. *Cancer Res* **68**, 5591-5598, doi:10.1158/0008-5472.CAN-08-0025 (2008).

22 Green, G. H. & Diggle, P. J. On the operational characteristics of the Benjamini and Hochberg False Discovery Rate procedure. *Stat Appl Genet Mol Biol* **6**, Article27, doi:10.2202/1544-6115.1302 (2007).

23 Peng, W. *et al.* Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. *Sci Transl Med* **4**, 118ra111, doi:10.1126/scitranslmed.3002629 (2012).

24 Xia, J. *et al.* INMEX--a web-based tool for integrative meta-analysis of expression data. *Nucleic Acids Res* **41**, W63-70, doi:10.1093/nar/gkt338 (2013).

25 Gallinetti, J., Harputlugil, E. & Mitchell, J. R. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem J* **449**, 1-10, doi:10.1042/BJ20121098 (2013).

26 Kennedy, B. K. & Lamming, D. W. The Mechanistic Target of Rapamycin: The Grand ConducTOR of Metabolism and Aging. *Cell Metab* **23**, 990-1003, doi:10.1016/j.cmet.2016.05.009 (2016).

27 Xu, S., Cai, Y. & Wei, Y. mTOR Signaling from Cellular Senescence to Organismal Aging. *Aging Dis* **5**, 263-273, doi:10.14336/AD.2014.0500263 (2014).

28 Hay, N. & Sonenberg, N. Upstream and downstream of mTOR. *Genes Dev* **18**, 1926-1945, doi:10.1101/gad.1212704 (2004).

29 Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471-484, doi:10.1016/j.cell.2006.01.016 (2006).

30 Longo, V. D. *et al.* Interventions to Slow Aging in Humans: Are We Ready? *Aging Cell* **14**, 497-510, doi:10.1111/acel.12338 (2015).

31 Masoro, E. J. Caloric restriction and aging: controversial issues. *J Gerontol A Biol Sci Med Sci* **61**, 14-19 (2006).

32 Cavallini, G., Donati, A., Gori, Z. & Bergamini, E. Towards an understanding of the anti-aging mechanism of caloric restriction. *Curr Aging Sci* **1**, 4-9 (2008).

33 Turturro, A., Hass, B. S. & Hart, R. W. Does caloric restriction induce hormesis? *Hum Exp Toxicol* **19**, 320-329 (2000).

34 Zhu, Z. *et al.* PI3K is negatively regulated by PIK3IP1, a novel p110 interacting protein. *Biochem Biophys Res Commun* **358**, 66-72, doi:10.1016/j.bbrc.2007.04.096 (2007).

35 Delacroix, L. *et al.* Cell-specific interaction of retinoic acid receptors with target genes in mouse embryonic fibroblasts and embryonic stem cells. *Mol Cell Biol* **30**, 231-244, doi:10.1128/MCB.00756-09 (2010).

36 Sever, R. & Glass, C. K. Signaling by nuclear receptors. *Cold Spring Harb Perspect Biol* **5**, a016709, doi:10.1101/cshperspect.a016709 (2013).

37 Lee, S. E. *et al.* Retinoid X receptor alpha overexpression alleviates mitochondrial dysfunction-induced insulin resistance through transcriptional regulation of insulin receptor substrate 1. *Mol Cells* **38**, 356-361, doi:10.14348/molcells.2015.2280 (2015).

38 Amigo, I. & Kowaltowski, A. J. Dietary restriction in cerebral bioenergetics and redox state. *Redox Biol* **2**, 296-304, doi:10.1016/j.redox.2013.12.021 (2014).

39 Chakrabarti, M. *et al.* Molecular Signaling Mechanisms of Natural and Synthetic Retinoids for Inhibition of Pathogenesis in Alzheimer's Disease. *J Alzheimers Dis* **50**, 335-352, doi:10.3233/JAD-150450 (2016).

40 Choi, B. K. *et al.* Reduction of ischemia-induced cerebral injury by all-trans-retinoic acid. *Exp Brain Res* **193**, 581-589, doi:10.1007/s00221-008-1660-x (2009).

41 Shen, H. *et al.* 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein. *J Neurosci Res* **87**, 545-555, doi:10.1002/jnr.21865 (2009).

42 Fusco, S. & Pani, G. Brain response to calorie restriction. *Cell Mol Life Sci* **70**, 3157-3170, doi:10.1007/s00018-012-1223-y (2013).

43 Meynet, O. & Ricci, J. E. Caloric restriction and cancer: molecular mechanisms and clinical implications. *Trends Mol Med* **20**, 419-427, doi:10.1016/j.molmed.2014.05.001 (2014).

44 Wu, C. W. & Storey, K. B. FoxO3a-mediated activation of stress responsive genes during early torpor in a mammalian hibernator. *Mol Cell Biochem* **390**, 185-195, doi:10.1007/s11010-014-1969-7 (2014).

45 van den Berg, M. C. & Burgering, B. M. Integrating opposing signals toward Forkhead box O. *Antioxid Redox Signal* **14**, 607-621, doi:10.1089/ars.2010.3415 (2011).

46 Obrochta, K. M., Krois, C. R., Campos, B. & Napoli, J. L. Insulin regulates retinol dehydrogenase expression and all-trans-retinoic acid biosynthesis through FoxO1. *J Biol Chem* **290**, 7259-7268, doi:10.1074/jbc.M114.609313 (2015).

47 Chiefari, E. *et al.* HMGA1 is a novel downstream nuclear target of the insulin receptor signaling pathway. *Sci Rep* **2**, 251, doi:10.1038/srep00251 (2012).

48 Araya, N. *et al.* Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. *J Biol Chem* **278**, 5427-5432, doi:10.1074/jbc.M210234200 (2003).

49 Daemen, S., Kutmon, M. & Evelo, C. T. A pathway approach to investigate the function and regulation of SREBPs. *Genes Nutr* **8**, 289-300, doi:10.1007/s12263-013-0342-x (2013).

50 Yuan, M., Pino, E., Wu, L., Kacergis, M. & Soukas, A. A. Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *J Biol Chem* **287**, 29579-29588, doi:10.1074/jbc.M112.386854 (2012).

51 van der Vos, K. E. & Coffer, P. J. FOXO-binding partners: it takes two to tango. *Oncogene* **27**, 2289-2299, doi:10.1038/onc.2008.22 (2008).

52 Robertson, L. T. *et al.* Protein and Calorie Restriction Contribute Additively to Protection from Renal Ischemia Reperfusion Injury Partly via Leptin Reduction in Male Mice. *J Nutr* **145**, 1717-1727, doi:10.3945/jn.114.199380 (2015).

Supplementary data

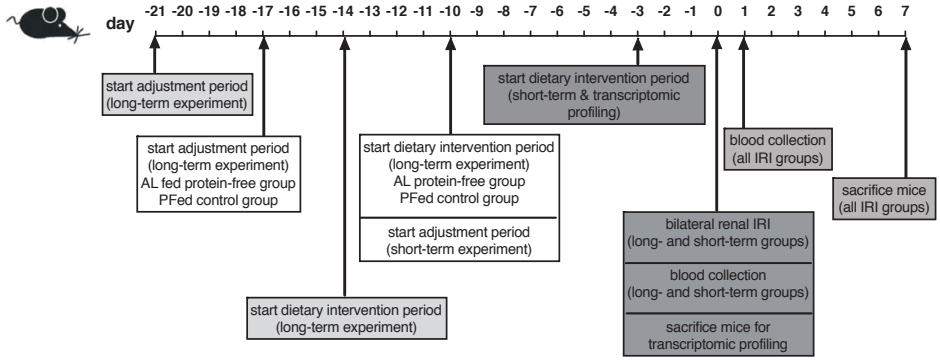


Figure S1. Schematic overview of the experimental design. After an acclimation period for all groups of 7 days on SDS chow, the long-term experiments started with diets given for either 10- or 14 days. At day -3 relative to induction of ischemia-reperfusion injury (IRI) the short-term dietary intervention and gene expression profiling experiments were started. At day 0, animals used for transcriptomic profiling were sacrificed for further analyses. In the other groups, blood collection was followed by induction of bilateral renal IRI for 37 minutes and collection of blood one day later. Mice were followed until day 7 after IRI, after which they were sacrificed.

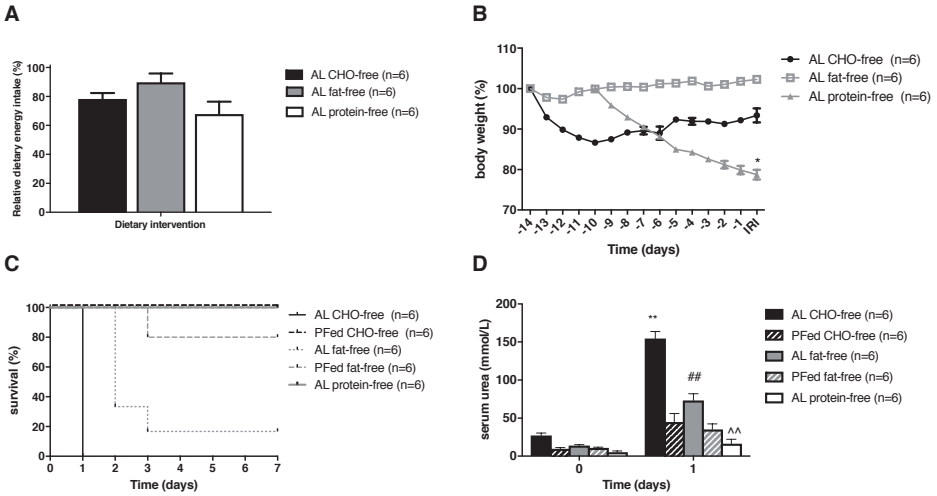


Figure S2. Effects on food intake, body weight and survival by macronutrient free diets and effects on kidney function upon subsequent renal ischemia reperfusion injury. **(A)** Relative to their control food intake during the 7 day acclimatization period, mice consumed (cumulatively during the whole intervention period) 21.7% less of the CHO-free diet, 10.1% of the fat-free diet and 32.7% of the protein-free diet. **(B)** Body weight of mice fed a CHO-free diet decreased during the 14-day period with 6.7%, while mice on a 14-day fat-free diet showed an increase in body weight with 2.3%. Mice fed a protein-free diet for 10 days lost more than 20% of their body weight on day 10. Because of this substantial loss of body weight, the protein-free diet was limited to a period of 10 days or less in the subsequent ischemia-reperfusion experiments. **(C)** Mouse mortality rates upon renal ischemia-reperfusion injury after both the CHO- and fat-free diet were significantly higher than their PFed controls ($P<0.05$). Mice fed a protein-free diet for 10 days showed a 100% survival following renal IRI. **(D)** Kidney function as determined by serum urea levels on day 1 after induction of renal IRI were significantly worsened in both the CHO-free ($P<0.05$) and fat-free ($P<0.05$) groups compared to PFed groups. Mice fed a protein-free diet showed lower serum urea levels one day after induction of IRI compared with CHO-free ($P<0.01$), the PFed CHO-free ($P<0.05$), the fat-free ($P<0.01$) and the PFed fat-free group ($P<0.05$). ** = $P<0.05$ compared to AL CHO-free diet on day 0. ## = $P<0.05$ compared to AL fat-free diet on day 0. ^^ = $P<0.05$ compared to AL protein-free diet on day 0.

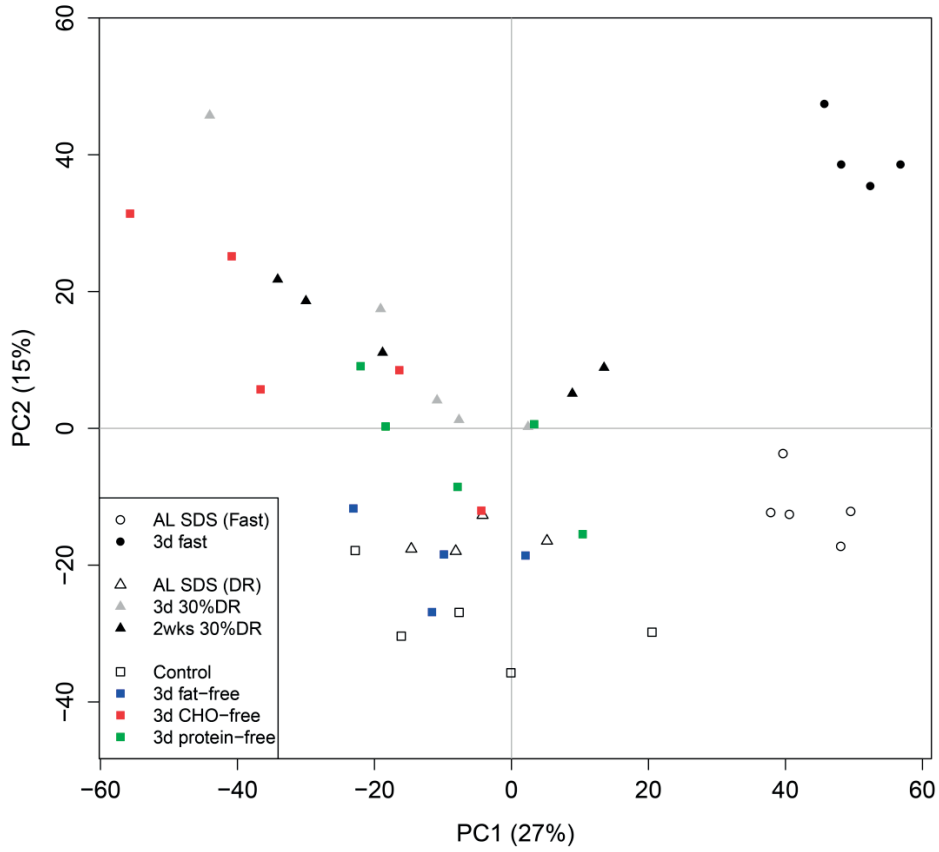


Figure S3. PCA of all probes on the microarray chip. This unbiased analysis shows the argest source of variability, PC1 is related to the date of hybridization. The 3-day fasting group and their corresponding control group were hybridized on a different date than the other groups. This result made the joint analysis of all datasets impracticable. PC2 explains only 15% of the variability, and it is related to the dietary interventions compared to the control samples. AL SDS (Fast)= control group of the 3-day fasting mice. AL SDS (DR) = control group of the 2 weeks 30% DR mice. Control = control group of the macronutrient-free diets.

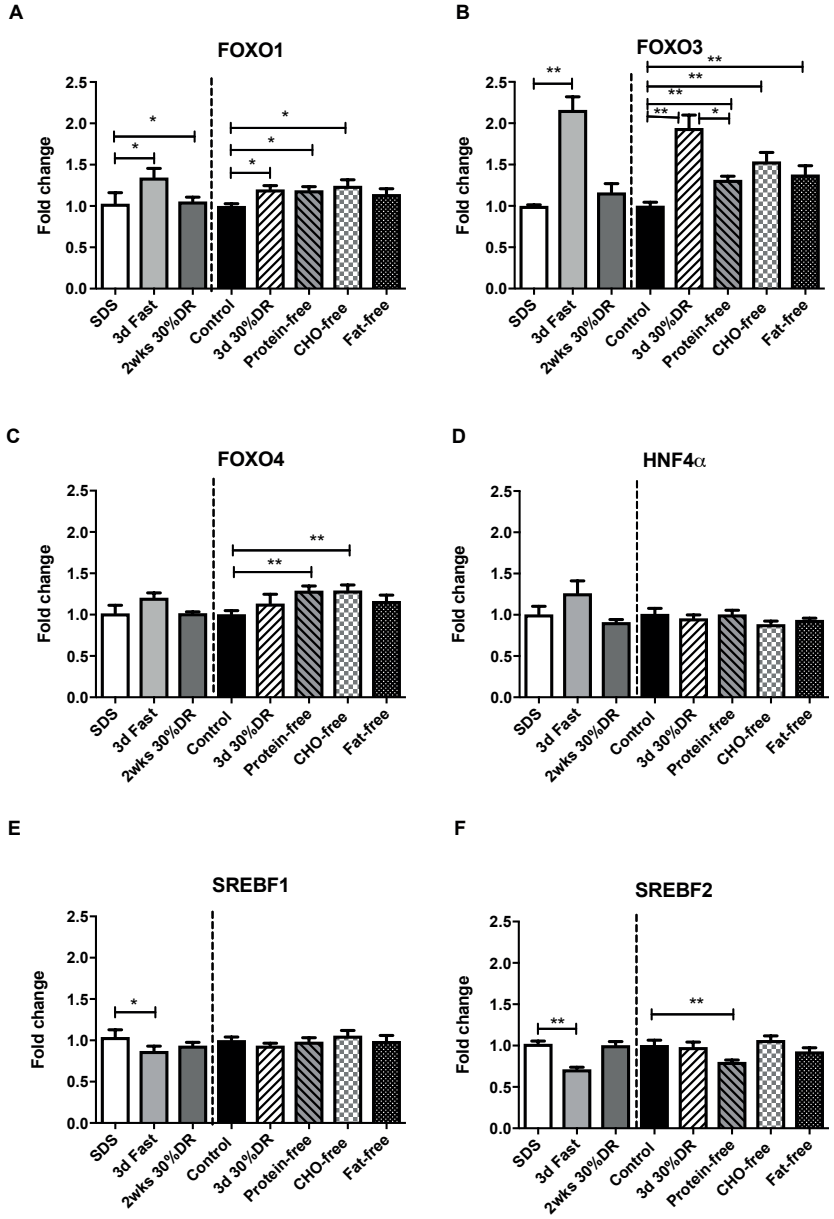


Figure S4. PCR data of mRNA expression levels of genes related to significantly regulated transcription factors. **(A)** FOXO1 was significantly higher after all diets except the fat-free diet. **(B)** FOXO3 showed significant up-regulation after all diets except the 2 weeks 30% DR. **(C)** FOXO4 expression levels were significant higher after three days of 30% DR and three days of a CHO-free diet. **(D)** HNF4α was not significantly regulated after any of the dietary interventions. **(E)** SREBF1 was down-regulated after three days of fasting, while **(F)** SREBF2 was significantly down-regulated after both three days of fasting and a 3-day protein-free diet. FOXO = forkhead box O; HNF4α = hepatocyte nuclear factor 4-alpha; SREBF = sterol regulatory element-binding transcription factor 1. * = P<0.05; ** = P<0.01.

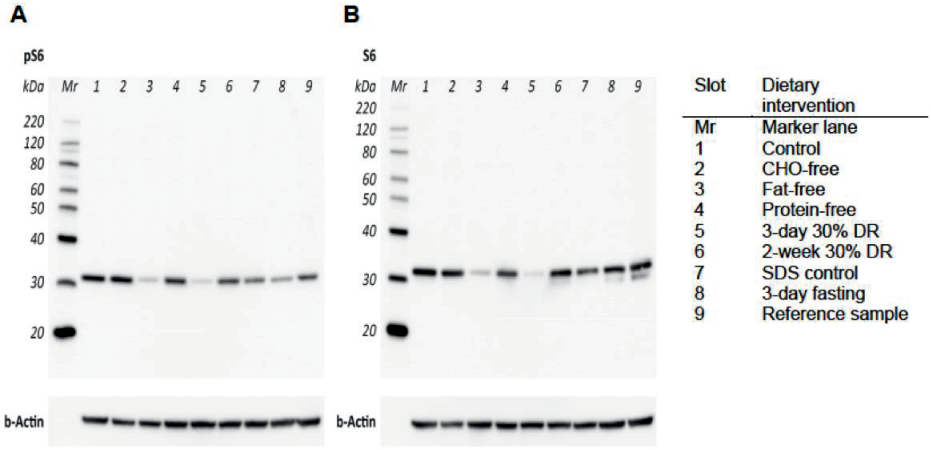


Figure S5. Representative Western blots of kidney extracts for both phosphorylated (A) and total (B) ribosomal protein S6 with β-actin as a loading control used for the relative quantification in Figure 4. The depicted exemplary blots show a full set of kidney extracts from each indicated diet group (lanes *1 – 8*) next to a marker lane (lane *Mr*) and a reference sample (lane *9*). In total, four such sets of Western blots were generated, each with four unique complete series for each diet group. For interblot comparison the exact same amount of the reference sample (lane 9) was loaded on each blot. The relative phosphorylation ratios depicted in Figure 4 are derived from the quantified signal of the main band in each lane, subtracted with background and corrected for both the sample dependent β-actin signal and the blot dependent signal of the reference sample.

Table S1. Composition and energy content of the individual diets.

Ingredient	Control	SDS-CRMP	Carbohydrate-free	Fat-free	Protein-free
Crude protein* (%/g)		16.4			
Casein, lactic (%/g)	20.0		99.2	31.3	0
Total kcal/g	3.8	3.3	3.4	3.5	3.8
Protein (g/kg)	179.0	183.5	887.9	280.1	0.0
Carbohydrate (g/kg)	710.0	574.0	1.0	710.0	889.0
Fat (g/kg)	45.0	33.6	45.0	0.0	45.0
Fiber (g/kg)	50.0	24.8	50.0	50.0	50.0
Protein (g%)	17.0	22.0	77.2	24.9	0
Carbohydrate (g%)	67.3	68.9	0.1	63.1	86.2
Fat (g%)	4.3	9.1	3.9	0.0	4.4
Fiber (g%)	4.7	4.2	4.3	4.4	4.8
Protein (kcal/kg)	716	734	3551	1121	0
Carbohydrate (kcal/kg)	2840	2296	4	2840	3556
Fat (kcal/kg)	405	303	405	0	405
Protein (kcal%)	18	22	90	28	0
Carbohydrate (kcal%)	72	69	0	72	90
Fat (kcal%)	10	9	10	0	10

***protein sources: wheat, barley, soya, maize, potato protein**

Table S2. Overview of the dietary interventions, the groups and numbers of mice used for phenotypical and transcriptional endpoints.

(A) Overview IRI long-term experiments

Dietary Intervention	Model	Duration (days)	Number of mice	Follow-up (days)	Parameters measured
Control SDS	C57BL/6	14	10	7	Survival, body weight, kidney function
Control	C57BL/6	14	12	7	Survival, body weight, kidney function
Fat-free	C57BL/6	14	6	7	Survival, body weight, kidney function
Pair-fed fat-free	C57BL/6	14	6	7	Survival, body weight, kidney function
Carbohydrate-free	C57BL/6	14	6	1	Survival, body weight, kidney function
Pair-fed carbohydrate-free	C57BL/6	14	6	7	Survival, body weight, kidney function
Protein-free	C57BL/6	10	6	7	Survival, body weight, kidney function

(B) Overview IRI short-term experiments

Dietary Intervention	Model	Duration (days)	Number of mice	Follow-up (days)	Parameters measured
Control	C57BL/6	3	4	7	Survival, body weight, kidney function
Protein-free	C57BL/6	3	4	7	Survival, body weight, kidney function
AL Control (control to 3-day 30% DR)	C57BL/6	3	4	7	Survival, body weight, kidney function
3 days 30% DR	C57BL/6	3	6	7	Survival, body weight, kidney function

(C) Overview of gene transcription experiments

Dietary Intervention	Model	Duration (days)	Number of arrays	Follow-up (days)	Parameters measured
Control SDS (control 2-week 30% DR)	C57BL/6	14	4	0	Gene expression profiling in kidney tissue
2-week 30% DR	C57BL/6	14	5	0	Gene expression profiling in kidney tissue
Control SDS (control 3-day fasting)	C57BL/6	3	5		Gene expression profiling in kidney tissue
3-day fasting	C57BL/6	3	4	0	Gene expression profiling in kidney tissue
Control	C57BL/6	3	5	0	Gene expression profiling in kidney tissue
Protein-free	C57BL/6	3	5	0	Gene expression profiling in kidney tissue
Fat-free	C57BL/6	3	4	0	Gene expression profiling in kidney tissue
Carbohydrate-free	C57BL/6	3	5	0	Gene expression profiling in kidney tissue

Table S3. List of genes corresponding to the DEPS found in common between all dietary interventions combined.

Affymetrix ID	Entrez Gene ID	Symbol	Gene Name	Log Ratio
1415802_PM_at	20501	SLC16A1	solute carrier family 16 (monocarboxylate transporter), member 1	-0,488
1415828_PM_a_at	28146	SERP1	stress-associated endoplasmic reticulum protein 1	-0,639
1416414_PM_at	100952	EMILIN1	elastin microfibril interfacer 1	-0,623
1416630_PM_at	15903	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1,008
1417150_PM_at	15567	SLC6A4	solute carrier family 6 (neurotransmitter transporter), member 4	0,537
1418591_PM_at	58233	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	-0,709
1419040_PM_at	56448	Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22	1,033
1422974_PM_at	23959	NT5E	5'-nucleotidase, ecto (CD73)	-1,037
1423392_PM_at	29876	CLIC4	chloride intracellular channel 4	-0,782
1423627_PM_at	18104	NQO1	NAD(P)H dehydrogenase, quinone 1	1,024
1424618_PM_at	15445	HPD	4-hydroxyphenylpyruvate dioxygenase	1,064
1424983_PM_a_at	78832	CACUL1	CDK2-associated, cullin domain 1	-0,746
1426568_PM_at	117591	SLC2A9	solute carrier family 2 (facilitated glucose transporter), member 9	0,684
1426896_PM_at	59057	ZNF24	zinc finger protein 24	-0,495
1427364_PM_a_at	18263	ODC1	ornithine decarboxylase 1	-0,975
1427931_PM_s_at	216134	PDXK	pyridoxal (pyridoxine, vitamin B6) kinase	1,251
1428332_PM_at	216505	PIK3IP1	phosphoinositide-3-kinase interacting protein 1	0,828
1433557_PM_at	52609	CBX7	chromobox homolog 7	0,618
1434437_PM_x_at	20135	RRM2	ribonucleotide reductase M2	-0,128
1434974_PM_at	228026	PDK1	pyruvate dehydrogenase kinase, isozyme 1	0,683
1436893_PM_a_at	57438	MARCH7	membrane-associated ring finger (C3HC4) 7, E3 ubiquitin protein ligase	0,585
1438211_PM_s_at	13170	DBP	D site of albumin promoter (albumin D-box) binding protein	1,768
1439797_PM_at	19015	PPARD	peroxisome proliferator-activated receptor delta	-0,838
1443870_PM_at	239273	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1,063
1449183_PM_at	12846	COMT	catechol-O-methyltransferase	-0,944
1449460_PM_at	142688	ASB13	ankyrin repeat and SOCS box containing 13	-0,686
1449848_PM_at	14675	GNA14	guanine nucleotide binding protein (G protein), alpha 14	-0,832

Table S3. List of genes corresponding to the DEPS found in common between all dietary interventions combined. (continued)

Affymetrix ID	Entrez Gene ID	Symbol	Gene Name	Log Ratio
1451122_PM_at	319554	IDI1	isopentenyl-diphosphate delta isomerase 1	-1,173
1452264_PM_at	209039	TNS2	tensin 2	0,757
1454709_PM_at	100201	TMEM64	transmembrane protein 64	-0,867
1455293_PM_at	235497	LEO1	LEO1 homolog, Paf1/RNA polymerase II complex component	-0,727
1455343_PM_at	233765	PLEKHA7	pleckstrin homology domain containing, family A member 7	0,922
1455393_PM_at	12870	CP	ceruloplasmin (ferroxidase)	1,037
1455490_PM_at	18703	PIGR	polymeric immunoglobulin receptor	0,924
1457689_PM_at	319934	SBF2	SET binding factor 2	0,761
1458176_PM_at	18628	PER3	period circadian clock 3	1,072
1460239_PM_at	66109	TSPAN13	tetraspanin 13	-0,925

The Afymmetrix probe sets, their corresponding Entrez Gene ID, symbol, gene name and log ratio are depicted in ascending order of the Affymetrix probe set. Only the probe sets with a corresponding gene ID are listed, therefore probe sets corresponding to the same gene ID are excluded. Log Ratio = log transformed fold change.

Table S4. Top 10 overrepresented canonical pathways after fasting, dietary restriction and macronutrient free diets individually ranked by their –log P-value.

3-day FASTING

Canonical Pathway	P-value	Genes Ratio
Superpathway Cholesterol Biosynthesis	3.41E-08	14/87 (16.1%)
Cholesterol Biosynthesis I / II / III	2.18E-06	8/40 (20.0%)
LXR / RXR Activation	2.20E-06	27/139 (19.4%)
NRF2-mediated Oxidative Stress Response	6.27E-06	34/195 (17.4%)
LPS/IL-1 Mediated Inhibition of RXR Function	7.46E-06	39/245 (15.9%)
Acute Phase Response Signaling	7.24E-05	30/181 (16.6%)
GADD45 Signaling	1.25E-04	8/24 (33.3%)
AMPK Signaling	1.25E-04	25/180 (13.9%)
VDR/RXR Activation	2.18E-04	17/88 (19.3%)
Xenobiotic Metabolism Signaling	3.00E-04	42/304 (13.8%)

2-week 30% DR

Canonical Pathway	P-value	Genes Ratio
Circadian Rhythm Signaling	1.41E-05	6/38 (15.8%)
Aldosterone Signaling Epithelial Cells	3.20E-05	11/168 (6.5%)
Guanosine Nucleotides Degradation II	7.85E-04	3/22 (13.6%)

Urate Biosynthesis	1.01E-03	3/22 (13.6%)
Phenylalanine Degradation IV	1.27E-03	3/39 (7.7%)
2-ketoglutarate Dehydrogenase Complex	1.48E-03	2/9 (22.2%)
Adenosine Nucleotides Degradation II	1.57E-03	3/26 (11.5%)
NRF2-mediated Oxidative Stress Response	2.39E-03	9/195 (4.6%)
Protein Ubiquitination Pathway	2.63E-03	11/270 (4.1%)
Purine Nucleotides Degradation II	2.71E-03	3/35 (8.6%)

3-day PROTEIN-FREE

Canonical Pathway	**P-value**	**Genes Ratio**
Intrinsic Prothrombin Activation Pathway	5.18E-04	4/37 (10.8%)
Superpathway Cholesterol Biosynthesis	6.79E-04	4/87 (4.6%)
LPS/IL-1 Mediated Inhibition of RXR Function	8.81E-04	10/245 (4.1%)
Mevalonate Pathway I	9.87E-04	3/29 (10.3%)
Creatine-phosphate Biosynthesis	1.77E-03	2/9 (22.2%)
Superpathway of Geranylgeranyl	2.02E-03	3/37 (8.1%)
PXR/RXR Activation	2.12E-03	5/92 (5.4%)
GADD45 Signaling	2.35E-03	3/24 (12.5%)
Tryptophan Degradation	4.84E-03	2/18 (11.1%)
Nicotine Degradation II	9.33E-03	4/85 (4.7%)

3-day CARBOHYDRATE-FREE

Canonical Pathway	**P-value**	**Genes Ratio**
Histamine Degradation	9.52E-05	6/29 (20.7%)
Pyrimidine Deoxygenase De Novo Biosynthesis I	1.57E-04	6/34 (17.6%)
Superpathway of Serine and Glycine Biosynthesis I	2.57E-04	4/18 (22.2%)
Glycolysis I	6.98E-04	7/41 (17.1%)
NRF2-mediated Oxidative Stress Response	8.17E-04	24/195 (12.3%)
Cholesterol Biosynthesis I / II / III	1.04E-03	5/40 (12.5%)
Colanic Acid Building Blocks Biosynthesis	1.52E-03	5/36 (13.9%)
Fatty Acid β-oxidation	1.52E-03	5/21 (23.8%)
Protein Ubiquitination Pathway	1.58E-03	30/270 (11.1%)
Folate Transformations I	1.84E-03	4/32 (12.5%)

3-day 30% DR

Canonical Pathway	**P-value**	**Genes Ratio**
Dopamine Degradation	1.08E-04	5/35 (14.3%)
Aryl Hydrocarbon Receptor Signaling	1.37E-04	9/140 (6.4%)
LPS/IL-1 Mediated Inhibition of RXR Function	2.48E-04	11/221 (5.0%)
PXR/RXR Activation	2.63E-04	6/65 (9.2%)
Circadian Rhythm Signaling	1.03E-03	4/33 (12.1%)

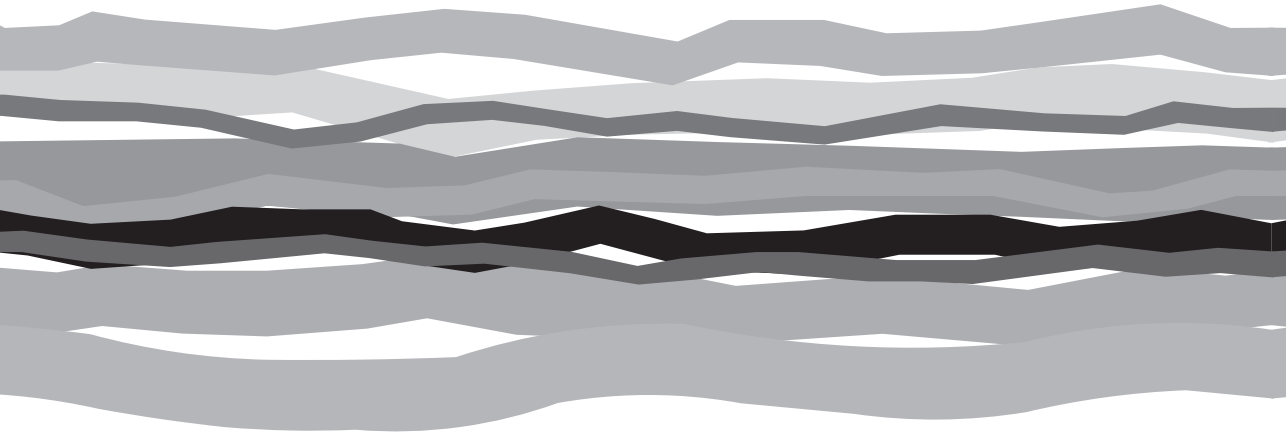
NRF2-mediated Oxidative Stress Response	1.42E-03	9/193 (4.7%)
Tyrosine Degradation I	1.82E-03	2/5 (40.0%)
Histamine Degradation	2.09E-03	3/19 (15.8%)
Noradrenaline and Adrenaline Degradation	2.14E-03	4/40 (10.0%)
Adipogenesis Pathway	2.52E-03	7/134 (5.2%)

Top 10 overrepresented pathways after dietary restriction and macronutrient free diets, with the exception of the 3-day fat-free diet that showed no differentially expressed probe sets. Analysis revealed no pathways regulated in common between the three protective diets. Furthermore, no pathways were oppositely regulated in the non-protective CHO-free diet. Genes ratio is the number and percentage of genes differentially expressed in ratio to the total number of genes involved in the pathway.

Table S5. Top 10 overrepresented canonical pathways after the meta-analysis including fasting, dietary restriction and macronutrient free diets ranked by their –log P-value.

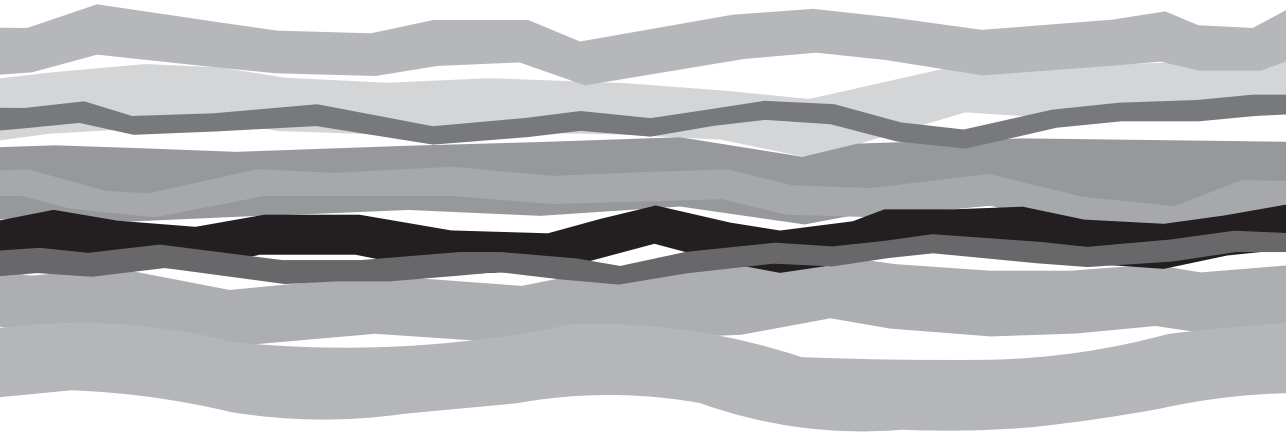
Meta-analysis II (Incl. 3-day 30% DR)				
Canonical Pathway	**Pathway Category**	**P-value**	**Genes Ratio**	**Z-score**
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	4.68E-05	10/221 (4.5%)	N/A
NRF2-mediated Oxidative Stress Response	Cellular Stress and Injury	8.92E-05	9/193 (4.7%)	N/A
PXR/RXR Activation	Nuclear Receptor Signaling	3.59E-04	5/65 (7.7%)	N/A
Noradrenaline and Adrenaline Degradation	Degradation/Utilization/ Assimilation; Hormones	5.25E-04	4.40 (10.0%)	N/A
Aryl Hydrocarbon Receptor Signaling	Cell Cycle Regulation; Apoptosis; Xenobiotic Metabolism, Nuclear Receptor Signaling	2.08E-03	6/140 (4.3%)	N/A
Superpathway of Cholesterol Biosynthesis	Fatty Acids and Lipids Biosynthesis, Sterol Biosynthesis	2.23E-03	3/28 (10.7%)	N/A
Glutathione-mediated Detoxification	Degradation/Utilization/ Assimilation; Detoxification	2.47E-03	3/29 (10.3%)	N/A
Circadian Rhythm Signaling	Neurotransmitters and Other Nervous System Signaling	3.59E-03	3/33 (9.1%)	N/A
Retinoate Biosynthesis I	Vitamin (A) Biosynthesis; Cofactors, Prosthetic Groups and Electron Carriers Biosynthesis	3.91E-03	3/34 (8.8%)	N/A
Dopamine Degradation	Degradation/Utilization/ Assimilation; Amines and Polyamines Degradation	4.25E-03	3/35 (8.6%)	N/A

The top 10 overrepresented pathways derived from the 279 DEPS in common between 3-days of fasting, 2 weeks 30% DR, three days of a protein-free and three days of a 30% DR diet. These pathways are mostly involved in regulation of nuclear receptor signalling (5 out of 10), biosynthesis signalling (2 out of 10) and cellular stress and injury (2 out of 10).



Chapter 6

Short-term absence of dietary protein or essential amino acids protects against hepatic ischemia-reperfusion injury



T.C. Saat[1]†, F. Jongbloed[1,2]†, S. van den Engel[1], J.L.A. Pennings[2], C.T. van Oostrom[2], J.N.M. IJzermans[1], H. van Steeg[2,3], M.E.T. Dollé[2], R.W.F. de Bruin[1]

† Authors contributed equally

Abstract

Ischemia reperfusion injury (IRI) is inevitable during major liver surgery and liver transplantation leading to oxidative stress. Preoperative short-term 30% dietary restriction (DR), three days of fasting and three days of a protein-free diet protect against IRI. Here, we further disentangled the role of essential amino acids on the effects of DR on liver IRI.

Male C57BL/6 mice were randomized to preoperative ad libitum control food or three days of 30% DR, methionine-free, leucine-free or tryptophan-free diet for three days. Liver IRI was induced by partial occlusion of the blood flow (70%) of the liver for 75 minutes. Food intake and body weight were monitored until postoperative day 1. Hepatic damage was measured biochemically and histologically at 6 and 24 hours after IRI. After completing each diet, liver gene expression profiles we determined by microarray analysis. All amino acid free diets resulted in body weight loss prior to IRI. Liver IRI was already significantly decreased by both the leucine- and tryptophan-free diets at 6 hours after IRI, and by the methionine-free diet at 24 hours. Microarray analysis showed similar transcriptomic responses in all amino acid-free diets, yet with a lower magnitude in the methionine-free diet. Detailed analysis of overlapping genes suggested a role for pathways involved in nuclear receptor signaling, stress resistance and cell cycle regulation via transcription factors NRF2, FOXM1, SREBF2 and SMARCB1.

A short-term preoperative tryptophan-free, leucine-free and methionine-free diet protect against hepatic IRI similar to a protein-free diet, through a network of pathways potentially activated by NRF2, FOXM1, SREBF2 and SMARCB1.

Introduction

Dietary restriction (DR) is defined as a reduction in food intake without malnutrition[1]. Long-term DR is known to extend lifespan[1], increase health span[2] and improve resistance to multiple stressors[1,3] in a wide variety of organisms[4-6]. Although the effect of DR on lifespan in humans is not known, studies show a favorable impact on metabolic parameters associated with long-term health[7-9]. In addition, previous studies showed that 2 weeks of 30% DR and three days of fasting increase stress resistance and protect against oxidative stress induced by ischemia reperfusion injury (IRI) in mice[1,10].

During major liver surgery and liver transplantation IRI is inevitable and a risk factor for complications including primary graft dysfunction and primary non-function, thereby causing morbidity and mortality[11-13]. Cessation of the blood flow (ischemia) leads to hypoxia, nutrient deprivation and activation of anaerobic metabolic systems[13]. Reperfusion of the liver promotes the activation of an inflammatory response, causing further cellular damage and the generation of reactive oxygen species[13]. Therefore, counteracting the adverse effects of IRI could improve the outcome after liver transplantation and liver resection. Unfortunately, at this moment no effective treatment for IRI is available.

Whether the protection by short-term DR is based on a reduction of calories or based on the reduction of specific nutrients is not yet completely unraveled. Also, the search for the underlying mechanisms of DR is still ongoing. In mice, liquid glucose supplementation did not interfere with the protective effect of 3 days of fasting against renal IRI[14]. In fruit flies, long-term protein restriction contributed more to extension of the life span compared to the reduction of carbohydrates or fat[15,16]. We previously investigated the role of specific macronutrients by unrestricted feeding of protein-, carbohydrate-, and fat free diets in inducing resistance against renal IRI. We showed that three days of a protein- but not a carbohydrate- or a fat-free diet before the induction of renal IRI improved survival and kidney function similarly as DR and fasting[17]. These results point towards a role for specific nutrients in the protective effect against renal IRI.

In the present study, we investigated the role of preoperative essential amino acid (EAA)-free diets by unrestricted feeding of methionine (Met)-free, leucine (Leu)-free and tryptophan (Trp)-free diets in reducing oxidative damage in the liver, and analyzed the transcriptomic response of the liver to these diets.

Material and methods

Animals

Male C57BL/6 (approximately 25 grams) were obtained from Harlan (Horst, the Netherlands). Animals were kept under standard laboratory conditions, and were housed in

individually ventilated cages (n = 3 animals/cage). All mice had ad libitum (AL) access to food and water (acidified with HCl). All experiments were performed with the approval of the appropriate local ethical board.

Diets

Before dietary intervention, all mice were acclimatized during 7 days and fed the standard Special Diet Services (SDS) chow. All other diets used for dietary intervention were purchased from Research Diets, Inc. (New Brunswick, NJ, USA). The SDS chow was used as a control diet for the fasted and 30% dietary restricted mice[1]. As a control for the protein-free and EEA-free diet, a specific control diet was used since this diet has the same protein source as the EAA-free diets, including all EAA (**Table S1**). The protein source of the EAA-free diets is derived from a crystalline L-amino acid mixture without one of the specific amino acids (Met-free, Leu-free or Trp-free). During the experiment, three days 30% DR was used as a negative control group while previous studies showed that this dietary intervention did not protect against IRI[17]. Mice on three days of 30% dietary restriction were given 70% of their normal daily intake of the SDS chow. Three days of protein-free diet was used as a positive control while this diet protects against IRI[12]. Mice on protein-free, Trp-free, Met-free and Leu-free diets were transferred to clean cages with the specific diet given at 4:00 pm. Mice on the control diet were used as the control group. Body weight of the mice was recorded daily. An overview of the composition of the diets used is shown in **Table S1**.

Hepatic ischemia reperfusion model

Mice were anesthetized by isoflurane/N2/O2 inhalation. To maintain their body temperature mice, were placed on a heating pad. All surgeries were performed between 9:00 and 1:00 pm. Partial (70%) hepatic IRI was induced by occlusion of the blood flow of the left lateral and median liver lobes with a non-traumatic micro vascular clamp for 75 minutes, which causes ischemia of the liver tissue. After clamp removal, the restoration of blood flow in the liver leads to reperfusion. No mortality was observed associated with this amount of damage to the liver. After surgery, all mice received 0.5 mL of phosphate-buffered saline subcutaneously and were placed under a heating lamp until they recovered from anesthesia. Directly after, all mice had free access to SDS chow and water.

Hepatocellular injury

Preoperatively fed and fasted mice were euthanized before surgery (baseline), 6 hours and 24 hours after reperfusion (n = 5-6 per time point). Serum levels of hepatic damage markers alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and lactate dehydrogenase (LDH) at the Central Clinical Chemical Laboratory of the Erasmus

University Medical Center. Hemorrhagic necrosis was scored before, and at 6 and 24 hours after reperfusion (n = 5-6 per time point) in 3 μm thick Hematoxylin and Eosin stained liver sections at a magnification of 100x by 2 observers blinded to the treatment. Hemorrhagic necrosis was characterized by the loss of the cellular architecture and the presence of erythrocytes in necrotic areas. The percentage of hemorrhagic necrosis per microscopic field was determined using the following scoring system: 0% (absent), 0% to 20% (<20% necrosis per microscopic field), 20% to 40% (20% to <40% necrosis per microscopic field) etcetera until 100% necrosis.

Immunohistochemistry

Frozen liver sections (5 μm) from different time points (baseline, 6 and 24 hours after reperfusion) were stained with a monoclonal antibody against neutrophils and visualized with an alkaline phosphatase secondary antibody. Per section two observers blinded to the treatment counted the number of neutrophils in 10 microscopic fields at magnifications of 400x.

Microarray analysis

For microarray analysis, mice were either fed the control diet or put on either a protein-free, Leu-free, Trp-free or Met-free diet for three consecutive days, after which the mice were sacrificed. Directly after each intervention, liver samples were taken and snap frozen in liquid nitrogen for further analyses. RNA isolation of the liver samples is as described previously17,18. RNA quality was tested via the RNA integrity number (RIN, range 0-10). All samples used scored a RIN between 7.7 and 9.5. Hybridization to Affymetrix HT MG-430 PM Array Plates was performed at the Microarray Department of the University of Amsterdam (the Netherlands), according to Affymetrix protocols and as described previously. The procedure for all samples was performed on the same batch. Normalization was done using the Robust Multichip Average (RMA) algorithm at the website www.arrayanalysis.org. Data output consisted of 45141 probes, whereby more probes could present the same Gene ID.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Differences in groups were analyzed by Mann-Whitney U tests with SPSS (version 21). Differences were considered significant at $P<0.05$. Microarray analyses were performed using the free software package R (R foundation), using the Linear Models for Microarray Data (limma) method with correction for multiple testing using the false discovery rate (FDR) according to Benjamini and Hochberg. Fold changes were expressed as the geometric mean per diet group against the corresponding ad libitum fed control group. A FDR <5% with fold change ≥1.5 were used as cut-off values for significance. The enrichment factor (EF),

defined as the number of times an observation was higher than expected by chance, was calculated via the formula: EF=nAB/((nA×nB)/nC), where nA is the number of differentially expressed probe sets (DEPS) in experimental group A, nB the number of DEPS in experimental group B, nC the number of total genes in the microarray, and nAB the number of common DEPS between nA and nB. Functional annotation and analyses were performed QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Subsequent pathway and target categories were generated using the QIAGEN's Ingenuity Target Explorer. The prediction inhibition or activation of the upstream transcription regulators are calculated via de statistical z-score based on the observed gene expression changes in our dataset. Calculating the z-score reduces the chance of significant predictions based on random data (http://ingenuity.force.com/ipa/articles/Feature_Description/ Upstream-Regulator-Analysis). A z-score ≥ 2 or ≤-2 was set as significant.

Results

Single EAA deprivation protects against hepatic IRI

To disentangle the role of single EAA in the protection against hepatic IRI, mice were administered a Met-free, Leu-free or Trp-free diet for three days. The results were compared with a control diet and diets that are known to either protect or not protect against IRI[17]. We have demonstrated that three days of a protein-free diet induces protection against renal IRI, while others also showed these results in other IRI models, including hepatic IRI[1]. On the contrary, three days of 30% DR does not induce resistance against renal IRI[17], which makes it a control group for reduced calorie intake and concomitant body weight loss observed during feeding of EAA free diets. Mice fed three days of a protein-free diet lose on average 9.8% of their body weight, while mice fed the non-protective three days of 30% DR showed a similar pattern in body weight loss (**Figure 1A**). Mice fed a single EAA-free also lost body weight: a Met-free diet resulted in 9.5% body weight loss, a Leu-free diet 6.2% and a Trp-free diet 8.5% (**Figure 1B**). Mice fed a protein- or EAA-free diet during three days restricted themselves on calorie intake (**Figure 1C**). Mice fed the Met-free diet restricted food intake by 44%, while the Leu-free diet resulted in a restriction of 45%. A Trp-free diet resulted in the smallest decrease in calorie intake, namely an average of 9% fewer calories than mice fed a control diet.

After induction of hepatic IRI, mice on a protein-free diet resumed intake of the control food within 24 hours and showed no further weight loss, while ad libitum fed mice or mice restricted for 30% during three days lost weight after surgery. Mice fed the EAA-free diet resumed food intake postoperatively and stabilized their body weight (**Figure 1B**).

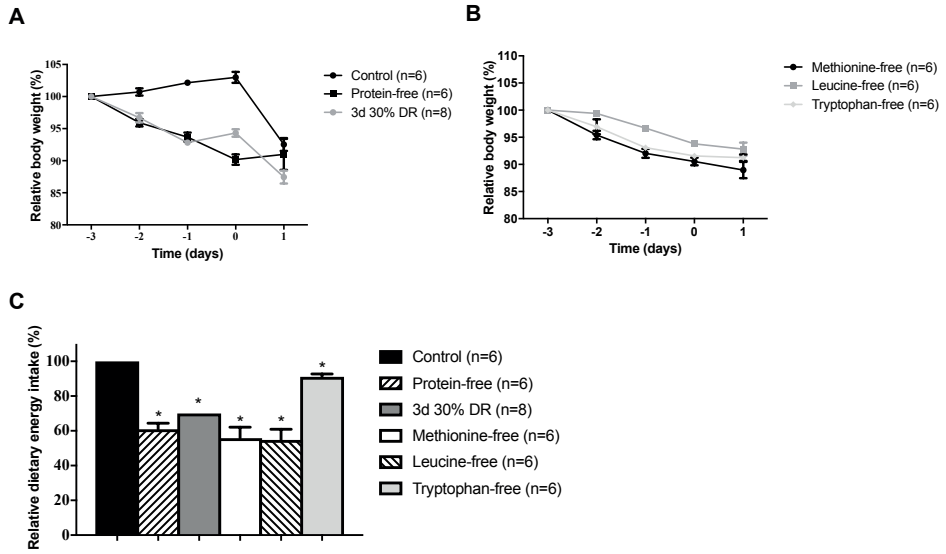


Figure 1. Body weight and food intake. **(A+B)** The nutritional interventions started at day -3 and hepatic IRI was induced at day 0. During the dietary intervention all mice, except the control chow fed group, showed a decrease in body weight. Postoperative day 1, mice fed an essential amino acid (EAA)-free diet lost less body weight compared to the control mice. Besides that, the fasted and protein-free mice gained weight. **(C)** Relative dietary energy intake during the 3-day intervention. Mice fed a tryptophan-free consumed on average 9% fewer calories than mice fed the control diet. Mice fed a methionine-free diet had 44.2% fewer calories, while mice on a leucine-free diet consumed 45.2% fewer calories than mice fed the control diet. In comparison, mice fed a protein-free diet ate 39.1% fewer calories.

Serum levels of liver enzymes ALAT (**Figure 2A**) and LDH (**Figure 2B**) were significantly lower in the Leu-free and Trp-free groups than in the control group at 6 and 24 hours postoperatively. At 24 hours after hepatic IRI, all EAA-free diets showed significantly lower liver enzymes (**Figure 2AB**). Histologic analysis of the livers supports these findings. At 6 hours after reperfusion, the percentage of hemorrhagic necrosis was significantly lower in the Leu-free and Trp-free diets than in the control group (**Figure 2C**). At 24 hours postoperatively, the percentage of necrosis was increased compared to 6 hours, but still a significant lower percentage of necrosis was seen in the Leu-free and Met-free diet, while the reduction did not reach significance in het Trp-free diet.

The number of neutrophils, as a marker for the acute inflammatory response in the liver, was significantly higher in het Leu-free and Trp-free diets than in the control group at 6 hours (**Figure 2D**), despite the lower percentage of hemorrhagic necrosis. Livers from animals fed the non-protective three days of 30% DR showed a significantly increased number of neutrophils, suggesting an ongoing inflammatory response after IRI in this group (**Figure 2D**).

Taken together, deprivation of essential amino acids methionine, leucine and tryptophan for three days induces protection against liver IRI similar to a 3-day protein-free

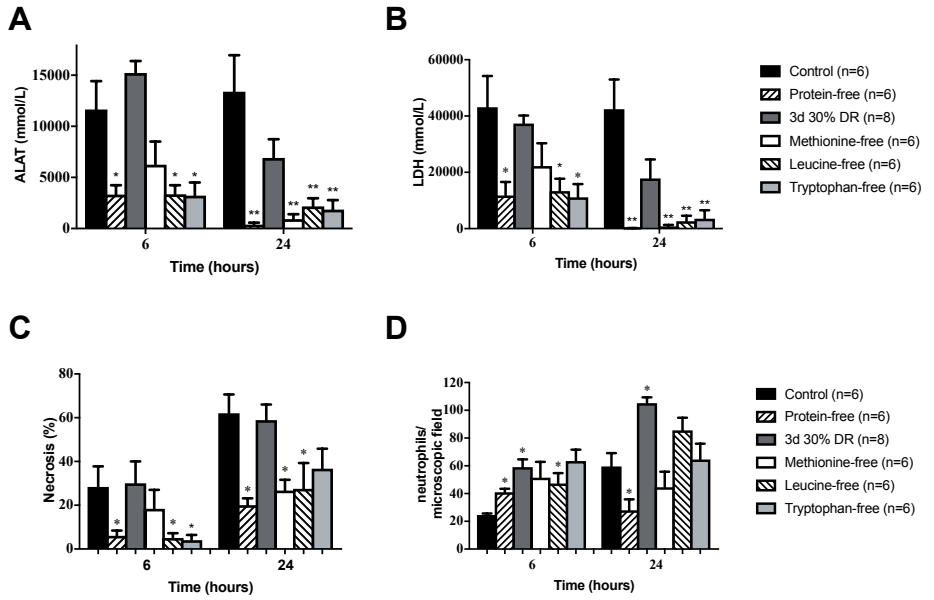


Figure 2. Essential amino acid free diets reduce liver injury. **(A)** Serum concentration of the liver-specific enzyme alanine aminotransferase (ALAT), indicative of liver damage, were significantly lower levels at 6 and 24 hours after IRI in mice fed a protein-free or amino acid free diet. **(B)** Twenty-four hours after IRI, serum concentration of lactate dehydrogenase (LDH), indicative of tissue breakdown, was significant lower in mice on a protein-free or amino acid free diet. *P<0.05, **P<0.01 versus the control group at the same time point. **(C)** Six hours post-reperfusion livers from mice fed leucine-free and tryptophan-free diets showed less hemorrhagic necrosis compared to the control group. Twenty-four hours post-reperfusion the fasted, protein-, leucine- and methionine-free diets induced significantly less damage compared to the control group. *P<0.05, **P<0.01 versus the control group. **(D)** At 24 hours post reperfusion, the protein-free diet had a lower number of neutrophils than the control group, while the 30% DR remained with a significantly higher neutrophil count. *P<0.05, versus the control group.

diet independent of preoperative body weight loss and voluntary DR. The beneficial effects of Leu and Trp are more pronounced at 6 hours after IRI while the effects of all EAA-free diets are seen at 24 hours postoperatively.

Transcriptional responses after a methionine-, leucine- and tryptophan-free diet are highly overlapping

To examine transcriptome responses amongst the different EAA-free diets and their controls, a principal component analysis (PCA) was made based on all probe sets in the microarray data (**Figure 3A**). In this unbiased PCA plot, PC1 explains 15% and PC2 explains 13% of the total variance of the probe sets. Examination of the PCA showed distinct separation of the mice in the control group and in the Met-free group, with uniformity within these groups along PC2. However, the Met-free cluster was grouped

closely on PC1 with the cluster of control mice. In contrast, the Leu-free and Trp-free groups showed a high degree of overlap, differentiating as a whole from the former two groups on PC1 and with an intermediate response on PC2 (**Figure 3A**). A dendogram, based on the unbiased hierarchical clustering of all mice, showed two distinct clusters of the control group and the Met-free group, which were connected higher up the dendogram (**Figure 3B**). The Leu-free and Trp-free groups had a similar hierarchy, with no distinctive clustering between the two groups.

The transcriptomic response was further analyzed by determining the differentially expressed probe sets (DEPS) after each dietary intervention compared to the control group. The Met-free diet induced the smallest number of DEPS, namely 572 with 348 up-regulated and 224 down-regulated DEPS. The Leu-free diet resulted in 933 DEPS, of which 476 were up-regulated and 457 down-regulated. The Trp-free diet induced 1155 DEPS of which 579 were up-regulated and 576 were down-regulated. To analyze the overlap between the groups, a Venn diagram was made with these DEPS, and 137 genes overlapped between all three diets (**Figure 3C**). The DEPS of the Leu-free and Trp-free diets showed the most similarity, with 603 genes present in both diets, comprising 65% of the DEPS induced by the Leu-free diet and 52% by the Trp-free diet. Of all differentially regulated genes in common between the Leu-free and Trp-free diets, 99% had the same directionality (results not shown). The Met-free induced the highest percentage of unique DEPS, namely 334 out of the 573 (58%). However, the directionality pattern of all differentially regulated genes reached an 84% agreement between Met-free and Leu-free diet and an 86% agreement between Met-free and Trp-free. Despite the stronger response induced by the Leu-free and Trp-free diets, there is a high overlap in transcriptional responses between all three EAA-free diets.

Essential amino acid-free diets regulate the cellular stress response, nuclear signaling and cell cycle

Subsequent unbiased analysis of the gene expression profiles was performed via pathway analysis and upstream transcription factor analysis. Analyses of differentially regulated pathways by the EAA-free diets focused on canonical pathways with a positive or negative z-score, which indicates an activation or inhibition of the pathway, respectively. Three days of Met-free diet resulted in 11 regulated pathways, of which 8 activated and 3 inhibited. The most prominent activated pathways were involved in cellular stress and injury, cell cycle regulation and growth signaling. Inhibition occurred mainly of the nuclear receptor signaling pathway (**Table 1A**). The Leu-free diet induced 29 differentially regulated pathways, of which 21 activated and 8 inhibited (**Table 1B**). These pathways represented mainly an activation of the cellular immune response and intracellular and second messenger signaling, in combination with an inhibition of nuclear receptor signaling and cellular stress and injury. Analysis of the Trp-free diet

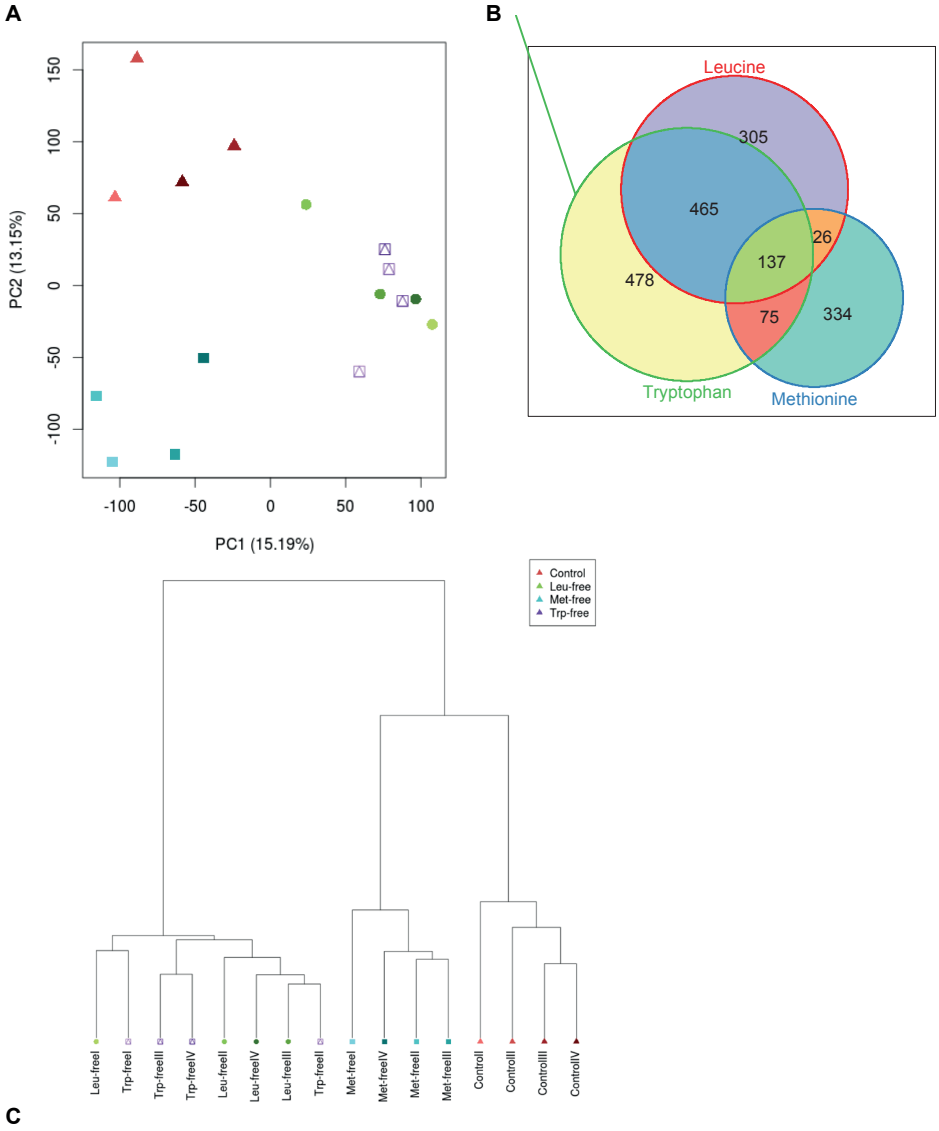


Figure 3 Principal component analysis and overlapping probe sets. **(A)** Unbiased principal component analysis (PCA) after normalization of all genes in the dataset. A distinct clustering of the control group and the methionine-free group is seen, with a clear separation of the groups on the principal component (PC) 2 but not on the PC1. The leucine-group and tryptophan-free have a highly overlapping clustering, with small intergroup variability. Their combined cluster is mainly separated from the control group and the methionine-free group on the PC1. **(B)** Dendogram based on the unbiased hierarchical clustering of all mice. The control group and methionine-free group have individual branches, but are connected higher up in the dendogram. The leucine-free and tryptophan-free group overlap and are intertwined in the dendogram. **(C)** Veen diagram of the differentially expressed probe sets (DEPS) overlapping between the methionine-free, leucine-free and tryptophan-free diets. A total of 137 genes overlapped between all diets. Of all genes in the leucine-free diet, 64% overlapped with the tryptophan-free diet. The methionine-free diet had the highest percentage of unique DEPS, namely 334 out of the 573 (58%).

revealed 15 pathways, including 8 activated and 7 inhibited pathways. The activated pathways were mostly involved in cellular immune responses and cell cycle regulation, while inhibited pathways included nuclear receptor signaling and cancer pathways (**Table 1C**). Comparison of overlapping pathways revealed that 5 out of the 11 pathways regulated by the Met-free diet were also regulated by the Leu-free diet, and 4 out of the 11 pathways by the Trp-free diet, all with similar directionalities. These overlapping pathways were amongst the highest regulated pathways including activation of NRF2-mediated stress response and G2/M Checkpoint Regulation pathways, and inhibition of the LPS/IL-1 mediated inhibition of RXR Function as well as the LXR/RXR activation pathway. In addition to the pathways in common between the three EAA-free diets, the Leu-free and Trp-free diets had another 4 pathways in common of which 2 had the same directionality, namely calcium-induced T-lymphocyte apoptosis and Sphingosine-1-phosphate signaling.

Table 1. Overview of the top overrepresented activated and inhibited canonical pathways after each amino acid-free diet ranked by their –log P-value.

A. Methionine-free

Canonical Pathway	**Pathway Classification**	**P-value**	**Genes Ratio**	**Z-score**
LXR/RXR Activation	Nuclear Receptor Signaling	3.24E-02	6/121 (5%)	-2.236
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	5.61E-10	22/222 (10.0%)	-1.897
Antioxidant Action of Vitamin C	Cellular Stress and Injury	1.61E-02	6/103 (5.8%)	-1.000
PPARa/RXRa Activation	Nuclear Receptor Signaling	8.78E-03	9/178 (5.1%)	+0.378
eNOS Signaling	Cardiovascular Signaling	3.37E-02	7/155 (4.5%)	+0.447
ERK5 Signaling	Intracellular and Second Messenger Signaling	3.53E-02	4/63 (6.3%)	+1.000
CNTF Signaling	Cytokine Signaling/ Neurotransmitters and Other Nervous System Signaling/ Cellular Growth	3.53E-02	4/63 (6.3%)	+1.000
UVA-Induced MAPK Signaling	Cellular Stress and Injury	1.54E-02	6/102 (5.9%)	+1.000
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	Cell Cycle Regulation	2.68E-03	5/49 (10.2%)	+1.342
Melanocyte Development and Pigmentation Signaling	Growth Factor Signaling	3.94E-02	5/95 (5.3%)	+2.000
NRF2-mediated Oxidative Stress Response	Cellular Stress and Injury	1.54E-07	18/193 (9.3%)	+2.646

B. Leucine-free

Canonical Pathway	Pathway Classification	P-value	Genes Ratio	Z-score
NRF2-mediated Odixative Stress Response	Cellular Stress and Injury	3.46E-05	18/193 (9.3%)	+2.309
Calcium-induced T-lymphocyte Apoptosis	Apoptosis/Cellular Immune Response	1.94E-04	9/66 (13.6%)	+1.000
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	2.09E-04	18/222 (8.1%)	-0.816
Role of NFAT in Regulation of the Immune Response	Intracellular and Second Messenger Signaling	6.87E-04	15/185 (8.1%)	+1.069
Cholecystokinin/Gastrin-mediated Signaling	Neurotransmitters and Other Nervous System Signaling	1.18E-03	10/101 (9.9%)	+1.265
CD28 Signaling in T Helper Cells	Cellular Immune Response	2.61E-03	11/131 (8.4%)	+0.447
PKCo Signaling in T Lymphocytes	Cellular Immune Response	2.77E-03	11/132 (8.3%)	+1.508
Tec Kinase Signaling	Intracellular and Second Messenger Signaling	6.95E-03	12/170 (7.1%)	+0.333
Phospholipase C Signaling	Intracellular and Second Messenger Signaling	7.42E-03	15/237 (6.3%)	+1.291
Thrombin Signaling	Cardiovascular Signaling	1.11E-02	13/203 (6.4%)	+0.905
LXR/RXR Activation	Nuclear Receptor Signaling	1.31E-02	9/121 (7.4%)	-1.000
CXCR4 Signaling	Cellular Immune Response / Cytokine Signaling	1.42E-02	11/165 (6.7%)	+0.378
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	Cell Cycle Regulation	1.76E-02	5/49 (10.2%)	+1.000
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Cellular Immune Response	1.77E-02	12/193 (6.2%)	-0.302
PI3K Signaling in B Lymphocytes	Cellular Immune Response	1.85E-02	9/128 (7.0%)	-0.333
G Beta Gamma Signaling	Intracellular and Second Messenger Signaling	1.96E-02	7/88 (8.0%)	+0.378
Relaxin Signaling	Organismal Growth and Development / Growth Factor Signaling	2.05E-02	10/152 (6.6%)	-0.447
Colorectal Cancer Metastasis Signaling	Cancer	2.27E-02	14/247 (5.7%)	+0.577
Actin Nucleation by ARP-WASP Complex	Intracellular and Second Messenger Signaling	2.96E-02	5/56 (8.9%)	+1.342
Synaptic Long Term Potentiation	Neurotransmitters and Other Nervous System Signaling	3.36E-02	8/120 (6.7%)	+0.707
Dendritic Cell Maturation	Cytokine Signaling/ Cellular Immune Response	3.55E-02	11/190 (5.8%)	+1.897
ICOS-ICOSL Signaling in T Helper Cells	Cellular Immune Response	3.65E-02	8/122 (6.6%)	+1.890
Sphingosine-1-phosphate Signaling	Intracellular and Second Messenger Signaling	3.81E-02	8/123 (6.5%)	+1.414
Macropinocytosis Signaling	Cellular Immune Response	4.01E-02	6/81 (7.4%)	+1.000
UV-Induced MAPK Signaling	Cellular Stress and Injury	4.07E-02	4/42 (9.5%)	-1.000
Antioxidant Action of Vitamin C	Cellular Stress and Injury	4.14E-02	7/103 (6.8%)	-1.000

B. Leucine-free (*continued*)

Canonical Pathway	Pathway Classification	P-value	Genes Ratio	Z-score
RhoGDI Signaling	Intracellular and Second Messenger Signaling	4.40E-02	10/173 (5.8%)	-0.378
IL-8 Signaling	Cytokine Signaling	4.42E-02	11/197 (5.6%)	+1.508
ERK5 Signaling	Intracellular and Second Messenger Signaling	4.58E-02	5/63 (7.9%)	+0.447

C. Tryptophan-free

Canonical Pathway	Pathway Classification	P-value	Genes Ratio	Z-score
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	1.25E-05	23/222 (10.4%)	-1.667
NRF2-mediated Oxidative Stress Response	Cellular Stress and Injury	1.40E-05	21/193 (10.9%)	+1.941
Colorectal Cancer Metastasis Signaling	Cancer	4.77E-04	21/247 (8.5%)	-0.447
Sumoylation Pathway	Cellular Stress and Injury/ Transcriptional Regulation	9.90E-04	11/96 (11.5%)	+0.333
Calcium-induced T Lypmhocyte Apoptosis	Apoptosis/Cellular Immune Response	3.32E-03	8/66 (12.1%)	+1.134
LXR/RXR Activation	Nuclear Receptor Signaling	2.11E-03	12/121 (9.9%)	-0.447
Wnt/B-catenin Signaling	Cancer / Organismal Growth and Development	1.20E-02	13/169 (7.7%)	-0.333
Cholecystokinin/Gastrin-mediated Signaling	Neurotransmitters and Other Nervous System Signaling	1.42E-02	9/101 (8.9%)	-0.333
MIF Regulation of Innate Immunity	Cellular Immune Response	1.85E-02	5/41 (12.2%)	+1.342
IGF-1 Signaling	Growth Factor Signaling	1.90E-02	9/106 (8.5%)	+0.447
UVC-Induced MAPK Signaling	Cellular Stress Injury	2.04E-02	5/42 (11.9%)	-1.342
MIF-mediated Glucocorticoid Regulation	Cellular Immune Response / Nuclear Receptor Signaling	3.47E-02	4/33 (12.1%)	+2.000
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	Cell Cycle Regulation	3.69E-02	5/49 (10.2%)	+2.000
Mitotic Roles of Polo-Like Kinase	Cell Cycle Regulation	3.81E-02	6/66 (9.1%)	-2.000
TWEAK Signaling	Apoptosis / Cytokine Signaling	3.82E-02	4/34 (11.8%)	-1.000
Sphingosine-1-phosphate Signaling	Intracellular and Second Messenger Signaling	4.36E-02	9/123 (7.3%)	+0.707

Pathways are defined via the canonical pathways as well as the pathway classification. Only pathways with a z-score were included. The canonical pathways and pathway category are based on the analyses done in Ingenuity and Ingenuity Target Explorer. Genes ratio = the number and percentage of probe sets differentially expressed in ratio to the total number of genes involved in the pathway. Z-score = based on the observed gene expression changes in the specific array datasets.

Analysis of the upstream transcription factors (TFs) activated or inhibited by the EAA-free diets resulted in a list of factors depicted in **Table S2**. The highest activated TF by the Met-free diet was NFE2L2, i.e. NRF2, followed by ATF4 and SMARCB1. NFE2L2 and

SMARCB1 were also significantly activated by the Leu-free and Trp-free diets, while ATF4 met a significant z-score in the Trp-free diet and almost significant in the Leu-free diet (1.838). The strongest inhibited TF by the Met-free diet was SREBF2, followed by FOXM1 and MYBL2. Both SREBF2 and FOXM1 were also significantly inhibited by the Leu-free and Trp-free diets, while MYBL2 only reached significance in the after the Leu-free (-2.000) and not in the Trp-free diet (-1.633) (**Table 2**). Of the 18 significantly regulated TFs by the Met-free diet, 14 and 13 TFs were also regulated by the Leu-free and Trp-free diet, respectively. All of these TFs showed the same directionality in all three EAA-free diets.

Table 2. List of significantly up-or down-regulated upstream transcription factors in the liver with corresponding z-scores regulated by all three amino-acid-free diets.

		Methionine-free		Tryptophan-free		Leucine-free	
Upstream Regulator	**State**	**Z-score**	**P-value**	**Z-score**	**P-value**	**Z-score**	**P-value**
NFE2L2	Activated	4.545	6.28E-13	2.975	3.12E-11	2.919	3.92E-07
SMARCB1	Activated	2.026	9.12E-06	2.436	9.74E-04	2.465	2.14E-06
SREBF2	Inhibited	-2.804	1.26E-04	-5.540	8.96E-26	-5.159	1.87E-28
FOXM1	Inhibited	-2.671	1.66E-04	-3.096	1.76E-05	-2.391	6.91E-06

Upstream regulator analysis of the differentially regulated genes found in common after 3-days of methionine-free, tryptophan-free and leucine-free diet revealed 4 significantly regulated transcription factors , of which 2 were activated and 2 were inhibited.

Discussion

In this study we demonstrated that, in addition to a protein-free diet, a Met-free, a Leu-free and a Trp-free diet for three days induce a similar protection against hepatic IRI. We show that cellular stress-, nuclear receptor-, and cell cycle regulation pathways are regulated by all three EAA-free diets, and that transcription factors NRF2, FOXM1, SREBF2 and SMARCB1 may be implicated in orchestrating this response.

Previously, we have shown that 2 weeks of 30% DR as well as three days of fasting are sufficient to induce resistance against hepatic and renal IRI[1,10,18]. Recently, we and others showed that absence of protein is the main contributor to these effects1[7,19]. Here, we further dissected the role of essential amino acids, and demonstrated that the absence of one single essential amino acid is sufficient to induce the increased stress resistance associated with DR leading to protection against hepatic IRI. Mice fed EAA-free diets voluntarily restricted food intake by approximately 30%. Since we showed that three days of 30% DR without deprivation of EAA is insufficient to induce protection against hepatic IRI, the restricted food intake due to EAA-free diets is ruled out as a factor and

the absence of EAA during three days is therefore responsible for inducing these beneficial effects.

All three EAA-free diets induced a similar protective effect, indicative of a common denominator of induction of the beneficial response. To extract this denominator, we determined mRNA expression profiles of liver tissue following administration of the EAA-free diets and performed microarray analyses. These data show that EAA deprivation modifies the transcriptome at three different pathway levels.

First, cell metabolism is altered as indicated by the nuclear receptor signaling response via the activation of the retinol pathway. Nuclear receptors are transcription factors that are known to be activated by steroid hormones and lipi-soluble agents, such as the retinoid acids (RAs)[20]. As previously shown[21,22], RAs able to induce many of the beneficial effects observed after DR. RAs are able to protect from ischemic stroke in the brain and have a positive effect on insulin resistance[23,24]. This activation is in line with our previous results, where nuclear receptor signaling activation was proposed as one of the main contributors to the beneficial effects of DR against renal IRI[17]. In our renal IRI model, upstream transcription factor FOXO3 was identified as a main player of the activation of the nuclear receptor response. FOXO3 was up-regulated after all three EAA-free diets, however only reached statistical significance after the Trp-free diet. Besides cell metabolism, FOXO3 phosphorylation and activation is at the basis of many processes, most importantly stress resistance and autophagy[25,26]. These data strengthen the role of nuclear receptor signaling mediated by FOXO3, however excludes FOXO3 as the only contributor for the beneficial effects. For instance, the LXR/RXR pathway has been implicated in the response as well[27]. The liver X receptor (LXR) is mainly expressed in the liver and is an important factor of its lipid metabolism[28]. However, we found an inhibition of this pathway in our EAA-free analysis. The down-regulation of the LXR/RXR pathway might in part be a response to the massive down-regulation of SREBF-2 by all EAA-free diets, which is a main regulator of the pathway[29]. Blocking SREBF-2 in a mouse model showed to be effective in preventing the development of hepatic steatosis and insulin resistance, and might be more important than the activation of the LXR/RXR pathway itself[27]. Our results suggest that inhibition of SREBF-2 might play a role in the protective effect on hepatic IRI. Future studies with for instance SREBF-2 -/- mouse models could highlight the effects of SREBF-2 deficiency on IRI.

Second, we found two transcription factors involved with inhibition of cellular proliferation. FOXM1, which increases G2/M DNA damage checkpoint activity[30], and SMARCB1 which inhibits cellular proliferation by mediating between various signaling pathways as part of the SWI/SNF complex. In recent work, SMARCB1 has been named often as a tumor suppressor via inhibition of cell growth[31,32]. Inhibition of cell growth is a common feature induced by DR regimens. We propose that the evolutionary adaptive response to DR that shifts resources from growth (metabolism and temporary withdrawal from

the cell cycle) to maintenance occurs already after three days of essential amino acid deprivation[33]. The associated increase in stress resistance is rapidly induced and offers robust protection against hepatic IRI.

Third, all three EAA-free diets induced a strong activation of the NRF2-mediated oxidative stress response, both on transcription factor and pathway level. NRF2 increases stress resistance[30], and we have already implicated NRF2 in the protection against renal IRI after three days of fasting[1,17,18]. In response to environmental stress, including protein deprivation, NRF2 is activated by phosphorylation of eIF2α via Gcn2[30,34,35] and results in the transcription of genes involved in antioxidant defense, reduction of inflammation and cell survival. In NRF2 knock-out models, similar effects of dietary restriction on cellular stress injury as in NRF2 proficient models could not be induced[30,36]. This indicates that NRF2 is indeed a major player yet not the only player in the protection against IRI. Our results demonstrate that activation of nuclear receptors signaling, inhibition of cellular proliferation and activation of the NRF2-pathway might be the essential package required to induce the beneficial effects of hepatic IRI. Validation studies should further emphasize on how these regulators induce protection against oxidative stress induced by IRI.

Taken together, we report that three essential amino acid-free diets given for three days protect against hepatic IRI in a mouse model possibly mediated via transcription factors NRF2, FOXM1, SREBF2 and SMARCB1. Further in depth analysis through functional and pharmacological modulation of these factors may provide mimetics for treatment against ROS-related injury.

References

1. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010;9:40-53.
2. Robertson LT, Mitchell JR. Benefits of short-term dietary restriction in mammals. Exp Gerontol 2013;48:1043-8.
3. Yamagishi T, Bessho M, Yanagida S, et al. Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts. Heart Vessels 2010;25:417-25.
4. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition 1989;5:155-71; discussion 72.
5. Mair W, Goymer P, Pletcher SD, Partridge L. Demography of dietary restriction and death in Drosophila. Science 2003;301:1731-3.
6. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009;325:201-4.
7. Ravussin E, Redman LM, Rochon J, et al. A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. J Gerontol A Biol Sci Med Sci 2015;70:1097-104.
8. Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science 2010;328:321-6.
9. Heilbronn LK, de Jonge L, Frisard MI, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 2006;295:1539-48.
10. Verweij M, van Ginhoven TM, Mitchell JR, et al. Preoperative fasting protects mice against hepatic ischemia/reperfusion injury: mechanisms and effects on liver regeneration. Liver Transpl 2011;17:695-704.
11. Uemura T, Randall HB, Sanchez EQ, et al. Liver retransplantation for primary nonfunction: analysis of a 20-year single-center experience. Liver Transpl 2007;13:227-33.
12. Ben-Ari Z, Issan Y, Katz Y, et al. Induction of heme oxygenase-1 protects mouse liver from apoptotic ischemia/reperfusion injury. Apoptosis 2013;18:547-55.
13. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. World J Gastroenterol 2013;19:1683-98.
14. Verweij M, van de Ven M, Mitchell JR, et al. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation 2011;92:752-8.
15. Jensen K, McClure C, Priest NK, Hunt J. Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in Drosophila melanogaster. Aging Cell 2015;14:605-15.
16. Bruce KD, Hoxha S, Carvalho GB, et al. High carbohydrate-low protein consumption maximizes Drosophila lifespan. Exp Gerontol 2013;48:1129-35.
17. Jongbloed F, Saat TC, Verweij M, et al. A signature of renal stress resistance induced by short-term dietary restriction, fasting, and protein restriction. Sci Rep 2017;7:40901.
18. Jongbloed F, de Bruin RW, Pennings JL, et al. Preoperative fasting protects against renal ischemia-reperfusion injury in aged and overweight mice. PLoS One 2014;9:e100853.
19. Solon-Biet SM, McMahon AC, Ballard JW, et al. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. Cell Metab 2014;19:418-30.
20. Sever R, Glass CK. Signaling by nuclear receptors. Cold Spring Harb Perspect Biol 2013;5:a016709.

21. Lee SE, Koo YD, Lee JS, et al. Retinoid X receptor alpha overexpression alleviates mitochondrial dysfunction-induced insulin resistance through transcriptional regulation of insulin receptor substrate 1. Mol Cells 2015;38:356-61.
22. Chakrabarti M, McDonald AJ, Will Reed J, Moss MA, Das BC, Ray SK. Molecular Signaling Mechanisms of Natural and Synthetic Retinoids for Inhibition of Pathogenesis in Alzheimer's Disease. J Alzheimers Dis 2016;50:335-52.
23. Choi BK, Kim JH, Jung JS, et al. Reduction of ischemia-induced cerebral injury by all-trans-retinoic acid. Exp Brain Res 2009;193:581-9.
24. Shen H, Luo Y, Kuo CC, et al. 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein. J Neurosci Res 2009;87:545-55.
25. Wu CW, Storey KB. FoxO3a-mediated activation of stress responsive genes during early torpor in a mammalian hibernator. Mol Cell Biochem 2014;390:185-95.
26. van den Berg MC, Burgering BM. Integrating opposing signals toward Forkhead box O. Antioxid Redox Signal 2011;14:607-21.
27. Rong X, Albert CJ, Hong C, et al. LXRs regulate ER stress and inflammation through dynamic modulation of membrane phospholipid composition. Cell Metab 2013;18:685-97.
28. Edwards PA, Kennedy MA, Mak PA. LXRs; oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. Vascul Pharmacol 2002;38:249-56.
29. Bensinger SJ, Bradley MN, Joseph SB, et al. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell 2008;134:97-111.
30. Hine CM, Mitchell JR. NRF2 and the Phase II Response in Acute Stress Resistance Induced by Dietary Restriction. J Clin Exp Pathol 2012;S4.
31. Kalimuthu SN, Chetty R. Gene of the month: SMARCB1. J Clin Pathol 2016;69:484-9.
32. Kohashi K, Oda Y. Oncogenic roles of SMARCB1/INI1 and its deficient tumors. Cancer Sci 2017.
33. Shanley DP, Kirkwood TB. Calorie restriction and aging: a life-history analysis. Evolution 2000;54:740-50.
34. Peng W, Robertson L, Gallinetti J, et al. Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. Sci Transl Med 2012;4:118ra11.
35. Lageix S, Rothenburg S, Dever TE, Hinnebusch AG. Enhanced interaction between pseudokinase and kinase domains in Gcn2 stimulates eIF2alpha phosphorylation in starved cells. PLoS Genet 2014;10:e1004326.
36. Martin-Montalvo A, Villalba JM, Navas P, de Cabo R. NRF2, cancer and calorie restriction. Oncogene 2011;30:505-20.

Table S1. Composition and energy content of the restricted and control diets.

Diet	*SDS*	*Control type I*	*Control type II*	*Carbohydrate-free*	*Fat-free*	*Protein-free*	*Methionine-free*	*Leucine-free*	*Tryptophan-free*
Ingredient	**g**	**g**	**g**	**g**	**g**	**g**	**g**	**g**	**g**
Casein, Lactic	0	200	0	992	313	0	0	0	0
L-Cystine	0	3	4.2	14.9	4.7	0	4.2	4.2	4.2
L-Isoleucine	0.77	0	7.6	0	0	0	7.6	7.6	7.6
L-Leucine	1.46	0	15.8	0	0	0	15.8	0	15.8
L-Lysine	1.04	0	13.2	0	0	0	13.2	13.2	13.2
L-Methionine	0.28	0	5.1	0	0	0	0	5.1	5.1
L-Phenylalanine	0.96	0	8.4	0	0	0	8.4	8.4	8.4
L-Threonine	0.69	0	7.2	0	0	0	7.2	7.2	7.2
L-Tryptophan	0.22	0	2.1	0	0	0	2.1	2.1	0
L-Valine	0.91	0	9.3	0	0	0	9.3	9.3	9.3
L-Histidine-HCl-H2O	0	0	4.6	0	0	0	4.6	4.6	4.6
L-Alanine	1.19	0	5.1	0	0	0	5.1	5.1	5.1
L-Arginine	0	0	6	0	0	0	6	6	6
L-Aspartic Acid	1.00	0	12.1	0	0	0	12.1	12.1	12.1
L-Glutamic Acid	3.72	0	38.2	0	0	0	38.2	38.2	38.2
Glycine	1.55	0	3	0	0	0	3	3	3
L-Proline	1.34	0	17.8	0	0	0	17.8	17.8	17.8
L-Serine	0.78	0	10	0	0	0	10	10	10
L-Tyrosine	0.69	0	9.2	0	0	0	9.2	9.2	9.2
Total L-Amino Acids		3	178.9	14.9	4.7	0	173.8	163.1	176.8
Corn Starch	42.37%	315	315	0	315	344	320.1	330.8	317.1

Table S1. Composition and energy content of the restricted and control diets. (continued)

Diet	*SDS*	*Control type I*	*Control type II*	*Carbohydrate-free*	*Fat-free*	*Protein-free*	*Methionine-free*	*Leucine-free*	*Tryptophan-free*
Maltodextrin 10		35	35	0	35	35	35	35	35
Sucrose		350	350	0	350	500	350	350	350
Cellulose, BW200	3.89%	50	50	50	50	50	50	50	50
Soybean Oil		25	25	25	0	25	25	25	25
Lard		20	20	20	0	20	20	20	20
Mineral Mix S10026		10	10	10	10	10	10	10	10
DiCalcium Phosphate		13	13	13	13	13	13	13	13
Calcium Carbonate		5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H2O		16.5	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix V10001		10	10	0	10	10	10	10	10
Vitamin Mix V10001C		0	0	1	0	0	0	0	0
Choline Bitartrate		2	2	2	2	2	2	2	2
Sodium Bicarbonate		0	7.5	0	0	0	7.5	7.5	7.5
FD&C Blue Dye #1		0	0	0	0.05	0	0.025	0.025	0
FD&C Yellow Dye #5		0.05	0.05	0	0	0	0	0.025	0.025
FD&C Red Dye #40		0	0	0.05	0	0.05	0.025	0	0.025
Total		1055.05	1038.45	1149.95	1124.75	1031.05	1038.45	1038.45	1038.45
kcal/g	**3.59**	**3.8**	**3.8**	**3.4**	**3.5**	**3.8**	**3.8**	**3.8**	**3.8**
g									
Protein	200.3	179.0	178.9	887.9	280.1	0.0	173.8	163.1	176.8

Table S1. Composition and energy content of the restricted and control diets. (continued)

Diet	*SDS*	*Control type I*	*Control type II*	*Carbohydrate-free*	*Fat-free*	*Protein-free*	*Methionine-free*	*Leucine-free*	*Tryptophan-free*
Carbohydrate	627.5	710.0	710.0	1.0	710.0	889.0	715.1	725.8	712.1
Fat	36.2	45.0	45.0	45.0	0.0	45.0	45.0	45.0	45.0
Fiber		50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
g%									
Protein	22.3	17.0	17.2	77.2	24.9	0	16.7	15.7	17.0
Carbohydrate	68.9	67.3	68.4	0.1	63.1	86.2	68.9	69.9	68.6
Fat	9.08	4.3	4.3	3.9	0.0	4.4	4.3	4.3	4.3
Fiber	4.23	4.7	4.8	4.3	4.4	4.8	4.8	4.8	4.8
kcal									
Protein	801	716	716	3551	1121.0	0	695	652	707
Carbohydrate	2510	2840	2840	4	2840.0	3556	2860	2903	2848
Fat	326	405	405	405	0.0	405	405	405	405
kcal%									
Protein	22	18	18	90	28.0	0	18	16	18
Carbohydrate	69	72	72	0	72.0	90	72	73	72
Fat	9.0	10	10	10	0.0	10	10	10	10

Control SDS = control diet given during acclimatization period and the diet of which the 3-day 30% DR diet is based. Control = control diet for the protein-free, methionine-free, leucine-free and tryptophan-free diet.

Table S2. List of significantly up-or down-regulated upstream transcription factors in the liver by a Methionine, Leucine or Tryptophane free diet with the corresponding z-scores

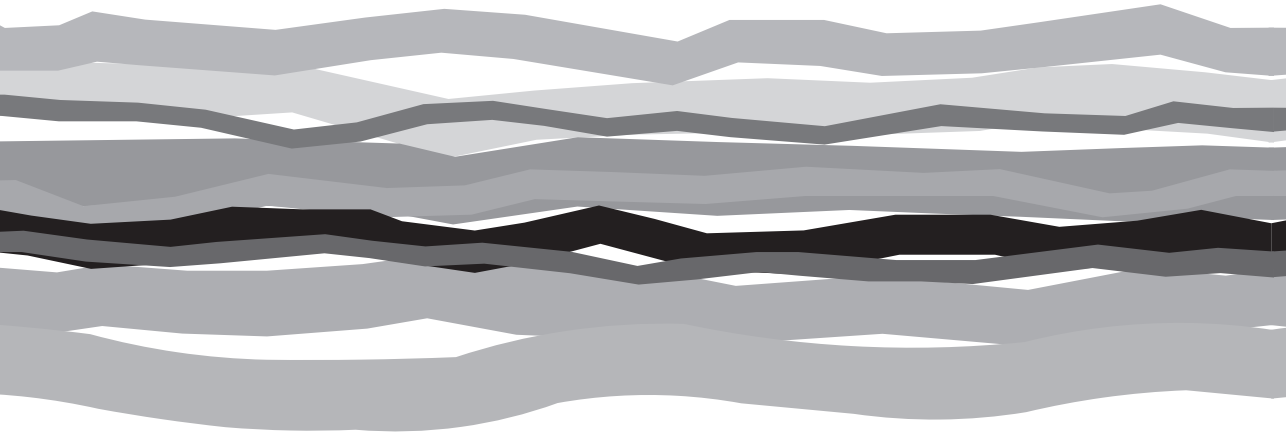
Methionine-free				Tryptophan		Leucine	
Upstream Regulator	**Gene ratio (Log2)**	**Z-score**	**P-value**	**Z-score**	**P-value**	**Z-score**	**P-value**
NFE2L2	0.297	4.545	6.28E-13	2.975	3.12E-11	2.919	3.92E-07
ATF4	0.393	2.911	7.45E-05	2.686	7.60E-09	1.838	2.41E-06
NUPR1	1.593	2.558	8.47E-04	2.846	2.92E-05	1.667	1.06E-05
KMT2D	0.180	2.449	1.15E-01	N/A	N/A	N/A	N/A
CEBPD	0.390	2.194	8.83E-04	1.994	3.42E-01	1.104	1.39E-02
NFKB1	0.007	2.182	1.37E-01	N/A	N/A	0.344	7.05E-03
KLF4	0.782	2.110	7.47E-02	1.994	4.08E-01	3.101	3.00E-01
SMARCB1	-0.164	2.026	9.12E-06	2.436	9.74E-04	2.465	2.14E-06
TAF4	0.393	2.000	2.57E-03	N/A	N/A	N/A	N/A
MAFF	1.398	2.000	3.37E-05	N/A	N/A	N/A	N/A
TBX2	0.047	-2.000	6.89E-02	-2.530	7.43E-04	-1.890	1.11E-02
Esrra	0.327	-2.000	4.14E-02	N/A	N/A	N/A	N/A
ATF6	0.216	-2.000	7.27E-03	-0.553	1.78E-05	-1.161	1.15E-04
FOXO1	-0.167	-2.139	9.30E-07	-0.465	1.51E-09	-1.269	6.53E-11
STAT5B	0.123	-2.157	2.12E-02	-0.901	3.17E-04	-1.463	4.42E-02
MYBL2	0.157	-2.236	1.79E-04	-1.633	1.79E-04	-2.000	1.03E-02
FOXM1	-0.080	-2.671	1.66E-04	-3.096	1.76E-5	-2.391	6.91E-06
SREBF2	0.086	-2.804	1.26E-04	-5.540	8.96E-26	-5.159	1.87E-28

Leucine-free				Tryptophan		Methionine	
Upstream Regulator	**Gene ratio (Log2)**	**Z-score**	**P-value**	**Z-score**	**P-value**	**Z-score**	**P-value**
KLF4	0.907	3.101	3.00E-01	1.994	4.08E-01	2.110	7.47E-02
NFE2L2	0.397	2.919	3.92E-07	2.975	3.12E-11	4.545	6.28E-13
KDM5B	-0.376	2.739	8.34E-08	2.999	3.88E-07	1.615	1.13E-04
TRIM24	-0.415	2.714	3.19E-04	3.043	1.20E-04	N/A	N/A
SMARCB1	-0.126	2.465	2.14E-06	2.436	9.74E-04	2.026	9.12E-06
POU5F1	-0.120	2.234	4.60E-01	-1.103	9.75E-04	N/A	N/A
ETS1	0.829	2.198	2.28E-02	N/A	N/A	N/A	N/A
NOTCH1	-0.165	2.137	8.04E-02	0.309	4.59E-02	0.727	9.18E-03
MYBL2	0.097	-2.000	1.03E-02	-1.633	1.79E-04	-2.236	1.79E-04
NFYA	-0.197	-2.007	1.27E-11	-0.840	5.51E-12	-0.128	7.02E-06
ARNTL	0.217	-2.180	2.56E-03	-1.539	1.41E-03	N/A	N/A
E2F2	-0.287	-2.236	5.13E-03	N/A	N/A	N/A	N/A
PDX1	0.029	-2.321	1.75E-05	-0.739	6.55E-06	N/A	N/A
FOXM1	-0.309	-2.391	6.91E-06	-3.096	1.76E-05	-2.671	1.66E-04

E2F1	-0.217	-2.480	1.50E-05	-1.068	6.08E-06	-1.564	8.48E-04
MLXIPL	-0.039	-2.510	1.27E-09	-2.345	1.35E-07	-0.927	3.18E-03
IRF7	-0.338	-2.599	2.83E-01	-3.088	1.15E-01	N/A	N/A
SIRT2	0.113	-2.646	8.82E-07	-2.646	3.30E-06	N/A	N/A
PPARGC1B	-0.681	-3.396	1.96E-07	-3.248	1.06E-05	-1.980	2.81E-02
SREBF1	-0.534	-4.605	2.68E-24	-4.998	1.55E-23	-1.020	6.88E-05
SREBF2	-0.453	-5.159	1.87E-28	-5.540	9.96E-26	-2.804	1.26E-04

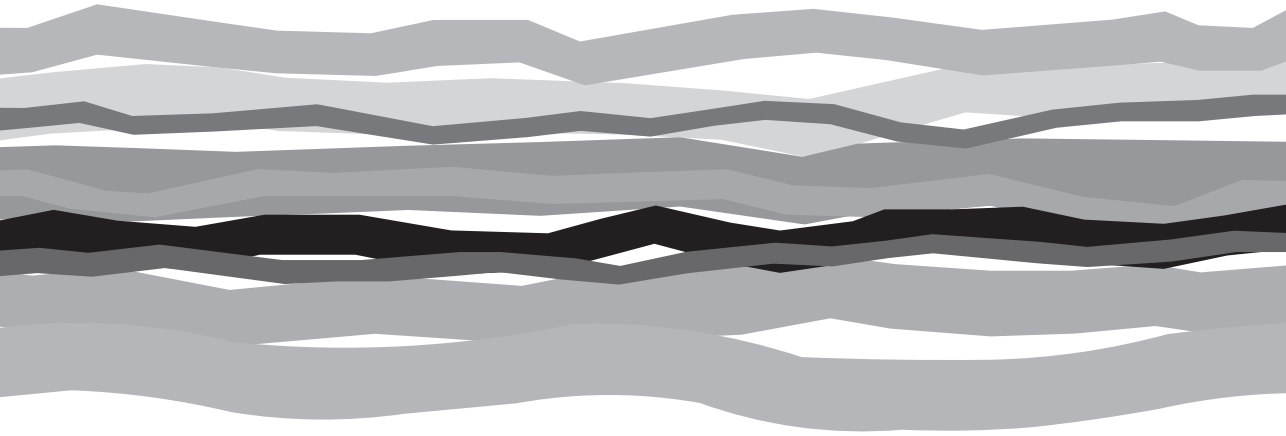
Tryptophan-free				Leucine		Methionine	
Upstream Regulator	**Gene ratio (Log2)**	**Z-score**	**P-value**	**Z-score**	**P-value**	**Z-score**	**P-value**
TRIM24	0.241	3.043	1.20E04	2.714	3.19E-04	N/A	N/A
KDM5B	-0.602	2.999	3.88E-07	2.739	8.34E-08	1.615	1.13E-04
NFE2L2	0.198	2.975	3.12E-11	2.919	3.92E-07	4.545	6.28E-13
NUPR1	0.772	2.846	2.92E-05	1.667	1.06E-05	2.558	8.47E-04
HTT	-0.347	2.784	1.12E-03	N/A	N/A	N/A	3.16E-04
ATF4	0.317	2.686	7.60E-09	1.838	2.41E-06	2.911	7.54E-05
SMARCB1	0.188	2.436	9.74E-04	2.465	2.14E-06	2.026	9.12E-06
HAND2	0.931	2.224	4.42E-02	N/A	N/A	N/A	N/A
FLI1	0.635	2.150	5.36E-05	1.364	6.55E-05	N/A	N/A
FOXO3	-0.209	2.095	2.00E-10	0.448	9.73E-08	0.588	1.75E-06
GATA4	-0.378	2.071	1.12E-01	N/A	N/A	N/A	N/A
TCF3	0.171	2.032	7.48E-05	1.265	9.72E-04	1.890	1.66E-02
IRF5	-0.327	-2.138	5.91E-03	N/A	N/A	N/A	N/A
STAT1	-0.661	-2.145	5.93E-05	-0.540	1.73E-06	-1.706	1.34E-03
MLX	-0.124	-2.181	1.20E-09	-1.977	1.09E-07	N/A	7.61E-03
MLXIPL	0.054	-2.345	1.35E-07	-2.510	1.27E-09	-0.927	3.18E-03
MYOD1	0.108	-2.465	1.20E-01	-1.242	1.85E-02	-1.657	5.12E-03
EGR1	-0.180	-2.520	2.14E-03	-1.925	7.99E-04	-1.344	3.23E-02
TBX2	-0.001	-2.530	7.43-04	-1.890	1.11E-02	-2.000	6.89E-02
SIRT2	0.170	-2.646	3.30E-06	-2.646	8.82E-07	N/A	N/A
IRF7	-0.349	-3.088	1.15E-01	-2.599	2.83E-01	N/A	N/A
FOXM1	-0.181	-3.096	1.76E-05	-2.391	6.91E-06	-2.671	1.66E-04
IRF3	0.254	-3.128	4.08E-03	-1.359	5.45E-04	N/A	N/A
PPARGC1B	-0.755	-3.248	1.06E-05	-3.396	1.96E-07	-1.980	2.81E-02
SREBF1	-0.247	-4.998	1.55E-23	-4.605	2.68E-04	-1.020	6.88E-05
SREBF2	-0.474	-5.540	8.96E-26	-5.159	1.87E-28	-2.804	1.26E-04

All significantly up-or down-regulated transcription factors (TF) after completion of each diet are shown, together with then log-ratio of the gene corresponding to the TF. Of all TF, the z-scores of the other two diets are compared and shown in the same table. N/A = not available.



Chapter 7

Fate and effect of intravenously infused mesenchymal stem cells in a mouse model of hepatic ischemia reperfusion injury and resection



Tanja C. Saat, Sandra van den Engel, Wendy Bijman-Lachger, Sander S. Korevaar, Martin J. Hoogduijn, Jan N.M. IJzermans, Ron W.F. de Bruin

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Abstract

Liver ischemia reperfusion injury (IRI) is inevitable during transplantation and resection, and is characterized by hepatocellular injury. Therapeutic strategies to reduce IRI and accelerate regeneration could offer major benefits. Mesenchymal stem cells (MSC) are reported to have anti-inflammatory and regeneration promoting properties. We investigated the effect of MSC in a model of combined IRI and partial resection in the mouse.

Hepatic IRI was induced by occlusion of 70% of the blood flow during 60 minutes, followed by 30% hepatectomy. $2x10^5$ MSC or PBS, were infused 2 hours before-, or 1 hour after IRI. Six-, 48- and 120 hours post-operatively mice were sacrificed. Liver damage was evaluated by liver enzymes, histology and inflammatory markers. Regeneration was determined by liver/body weight ratio, proliferating hepatocytes and TGF-β levels. Fate of MSC was visualized with 3Dcryo-imaging.

Infusion of $2x10^5$ MSC 2 hours before-, or 1 hour after IRI and resection showed no beneficial effects. Tracking revealed that MSC were trapped in the lungs, did not migrate to the site of injury and many cells had already disappeared 2 hours after infusion. Based on these findings we conclude that intravenously infused MSC disappear rapidly, and were unable to induce beneficial effects in a clinically relevant model of IRI and resection.

Introduction

The liver has the unique ability of regeneration as a response to liver injury. In healthy individuals the liver can compensate an acute loss of 70% and return to its original mass within 30 days of resection[1]. Thanks to this remarkable feature and advances in surgical techniques, large (oncologic) liver resections as well as split- and living donor liver transplantation are possible. However, in patients with chronic liver diseases, liver cirrhosis or malignancies, regeneration of the liver is often compromised due to chemotherapy[2], poor nutritional status[3] and the increasing age of patients[4]. Besides that, leaving a smaller fraction of residual liver after resection is a risk factor for postoperative morbidity due to hepatic dysfunction and infectious complications[5].

Liver transplantation (LTx) is the only life-saving treatment for end-stage hepatic diseases[6] and a treatment for patients with a primary liver tumor or colorectal metastases[7-9]. However, ischemia reperfusion injury (IRI), caused by interruptions of the hepatic blood flow, is inevitable during LTx and liver resection. Ischemia is characterized by ATP depletion and activation of anaerobic metabolic pathways, whereas reperfusion activates a cascade of pathways that causes further cellular damage and inflammation. IRI leads to a decreased regenerative capacity of hepatocytes, tissue necrosis, and apoptosis[10,11]. Liver IRI is the leading cause of hepatocellular injury causing morbidity and mortality after LTx and may negatively affect liver regeneration after both post mortal and living donor LTx[12-14]. Taken together, potential therapeutic strategies to reduce hepatic IRI and accelerate liver regeneration could offer major benefits in both LTx and resection.

Mesenchymal stem cells (MSC) are able to differentiate into different cell types, secrete growth factors, and have immunomodulatory and anti-inflammatory properties[15,16]. Therefore, MSC are considered as a potential therapy to prevent or ameliorate hepatic IRI and stimulate liver regeneration.

In rodents, MSC have the potential to reduce hepatic IRI by suppressing oxidative stress and inhibiting apoptosis[13,17,18]. Moreover, MSC improved liver regeneration in a resection model[13,19,20]. However, only few studies investigated the effect of MSC in a combined hepatic IRI and partial hepatectomy model[19,21-23], while this model is relevant for translation to the clinical setting. Results from these studies suggest that MSC have beneficial effects on both IRI and regeneration. However, the use of MSC in a large animal model showed inconsistent results on IRI[24,25]. Therefore, it remains unclear if and how MSC are able to prevent IRI and/or stimulate regeneration. The purpose of the present study is to investigate in a clinically relevant mouse model whether MSC are able to reduce hepatic IRI and stimulate liver regeneration after induction of hepatic IRI and partial liver resection.

Material and methods

Animals

Male C57BL/6 (age 10-12 weeks, ~25 g) were obtained from Harlan, (Horst, the Netherlands). Animals were kept under standard laboratory conditions, and housed in individually ventilated cages (n=3 animals/cage). The animals had free access to food and water (acidified with HCl). All experiments were performed with the approval of the institutional animal welfare committee (Protocol EMC2271).

Mesenchymal stem cell cultures

MSC were isolated from abdominal adipose tissue of C57BL/6 mice. Syngeneic mouse MSC were used to avoid xenogeneic and allogeneic responses. The tissue was mechanically disrupted and enzymatically digested with 0.5 mg/mL collagenase type IV (Life Technologies, Paisley, UK) in RPMI 1640 Medium with glutaMAX (Life technologies) for 30 min at 37 C° under continuous shaking. The obtained cell suspension was washed and plated in tissue culture flasks in MEM-α with 15% fetal calf serum, and 100U/mL pencillin and 100mg/mL streptomycin (1% p/s) (all Invitrogen, Germany). Non-adherent cells were removed after 2-3 days and subsequently culture medium was refreshed once a week. Plastic adherent cells were removed by trypsinization after reaching 70-80% confluency. Cells were maintained at 37°C, 5% CO_2, and 95% humidity. MSC of passage 3-5 were used for experiments. Before intravenous infusion of $2x10^5$ MSC in 200µl PBS, cells were filtered through a 40µm sieve. The phenotype of these MSC used in previous studies was confirmed as they expressed CD44 and Sca-1, were negative for CD11b and CD45, were capable of differentiating into osteoblasts and adipocytes, and had the ability to inhibit conA stimulated lymphocyte proliferation[15].

Surgical model

Mice were anesthetized by isoflurane/O_2 inhalation. To maintain their body temperature mice were placed on a heating pad. All surgeries were performed between 9:00am and 1:00pm. After a midline laparotomy, partial hepatic ischemia was induced by occlusion of the blood flow of the left lateral and median liver lobes with a non-traumatic micro vascular clamp for 60 minutes. The median and lateral lobe (approximately 70% of the liver) showed significant discoloration. After clamp removal, restoration of blood flow in the ischemic liver lobes causes reperfusion injury. Directly after removing the clamp, a partial hepatectomy (PH) was performed by resection of the left lateral liver lobe (approximately one-third of the liver). The abdomen was closed in two layers using Safil 5-0 (B.Braun, Germany). Mortality associated with this amount of damage to the liver was not observed. After surgery all mice received 0.5 mL of phosphate-buffered

saline subcutaneously and were placed under a heating lamp until they recovered from anesthesia. Directly after surgery, all mice had free access to food and water.

Our pilot study showed that administration of $1x10^5$ MSC or $3x10^5$ was ineffective, or showed a trend towards increased injury, respectively (**Figure S1**). Therefore, we chose a cell number of $2x10^5$ MSC as a potentially effective number of cells. We chose a pre-ischemic time point of administration (2 hours before) and a time point 1 hour after ischemia to determine the effect of administration before, and after inducing injury. Animals were treated intravenously via the tail vein with $2x10^5$ MSC in 200µl PBS or with PBS alone as vehicle control. Seven mice per group were infused 2 hours before hepatic IRI and resection, or 1 hour after removing the clamp. They were sacrificed 6 and 48 hours, and 5 days after surgery. Liver and blood samples were collected for further research. Control mice were sacrificed without undergoing any injection or intervention, their tissues were used as a baseline for mRNA expression levels.

Liver/body weight ratio

Mice were weighed daily. The resected liver lobe was weighed after PH. At time of sacrifice, livers were removed, and animals and livers were weighed. The liver/body weight ratio was calculated as the wet liver weight divided by the total body weight of the mouse.

Hepatocellular injury

Blood samples were collected at time of sacrifice 6 hours, 48 hours or 5 days after surgery (n=7 per time point). Sera were analyzed for alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) levels at the Central Clinical Chemical Laboratory of the Erasmus University Medical Center.

Hemorrhagic necrosis was scored in 3 µm thick paraffin liver sections stained with Hematoxylin and Eosin at a magnification of 100x by 2 independent observers blinded to the treatment (n=5 per time point). Hemorrhagic necrosis was characterized by the loss of the cellular architecture and the presence of erythrocytes in necrotic areas. The percentage of hemorrhagic necrosis per microscopic field was scored with the following scoring system: 0%, 1% to 20% (≤20% necrosis per microscopic field), 21% to ≤40% necrosis per microscopic field, 41% to ≤60% necrosis per microscopic field, 61% to ≤80% necrosis per microscopic field and 81% to 99% necrosis per microscopic field and 100% necrosis.

Immunohistochemistry

Frozen (5 µm) liver sections from mice infused with PBS or MSC and sacrificed 6 hours, 48 hours or 5 days after reperfusion and resection were stained with monoclonal antibodies against proliferating cell nuclear antigen (PCNA) (Abcam, UK). They were visualized

with a horseradish peroxidase-conjugated secondary antibody (DAKO, Belgium). In 8 microscopic fields per section, the number of positive cells was counted by two observers (TS, SvD) blinded to the treatment at magnifications of 200 to 400x.

Quantitative RT-PCR

Total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), purified by a DNase treatment (RQ1 RNase-Free DNase; Promega Benelux B.V., Leiden, the Netherlands), and reverse transcribed to complementary DNA using random hexameter primers and Superscript II RT (both from Invitrogen, Breda, the Netherlands) according to manufactures instructions. Quantitative real-time PCR was performed using a MyiQ single-color Real-Time PCR Detection System with SYBR Green incorporation (both from Bio-Rad Laboratories B.V., Veenendaal, the Netherlands; primer sequences are available upon request).

B2M and HPRT were used as housekeeping genes, IL-6 and TNF-α were used as genes indicative for inflammation. To assess cytoprotection we measured HO-1 and IL-10 as an anti-inflammatory genes.

TGF-β was used as a marker for regeneration. Primers used to amplify the genes of interest were obtained from Sigma, the Netherlands. The relative expression was calculated as $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$, corrected for expression levels in healthy control animals. Mice without any infusion and surgery were used as healthy controls. Each sample was tested at least in duplicate.

MSC tracking by 3D cryo-imaging

One batch of MSC was labeled with fluorescent Qtracker 605 beads according the manufacturer's description (stains viable MSC; Life Technologies, Grand Island, NY, USA). $2x10^5$ labeled MSC were infused in the tail vein directly after hepatic IRI. Two hours after the induction of IRI the mouse was sacrificed by isoflurane overdose and the whole mouse was snap frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, the Netherlands). 3D anatomical and molecular fluorescence videos were generated by CryoViz^{tm} (BioInVision, Mayfield village, OH, USA), allowing the detection of single labeled cells.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Differences between groups were analyzed by Mann-Whitney U tests using SPSS (version 21). Differences were considered significant at P values less than 0.05.

Results

Effect of mesenchymal stem cells on liver damage

Liver damage was evaluated by measuring serum transaminases and histological damage. 6 hours after reperfusion and partial hepatectomy (PH), serum ALAT (7400±1392 U/L) and ASAT (7529±1202 U/L) levels were significantly lower in mice infused with MSC 2 hours before IRI and PH compared with their PBS controls (ALAT 13271±1644 U/L) (ASAT 13207±1131 U/L) (*P*=0.015; **Figure 1A**) (*P*=0.005, **Figure 1B**). Mice infused with MSC 1 hour after reperfusion and PH showed no significant difference compared with their PBS control group (**Figure 1A, 1B**). 48 hours after reperfusion and PH, there were

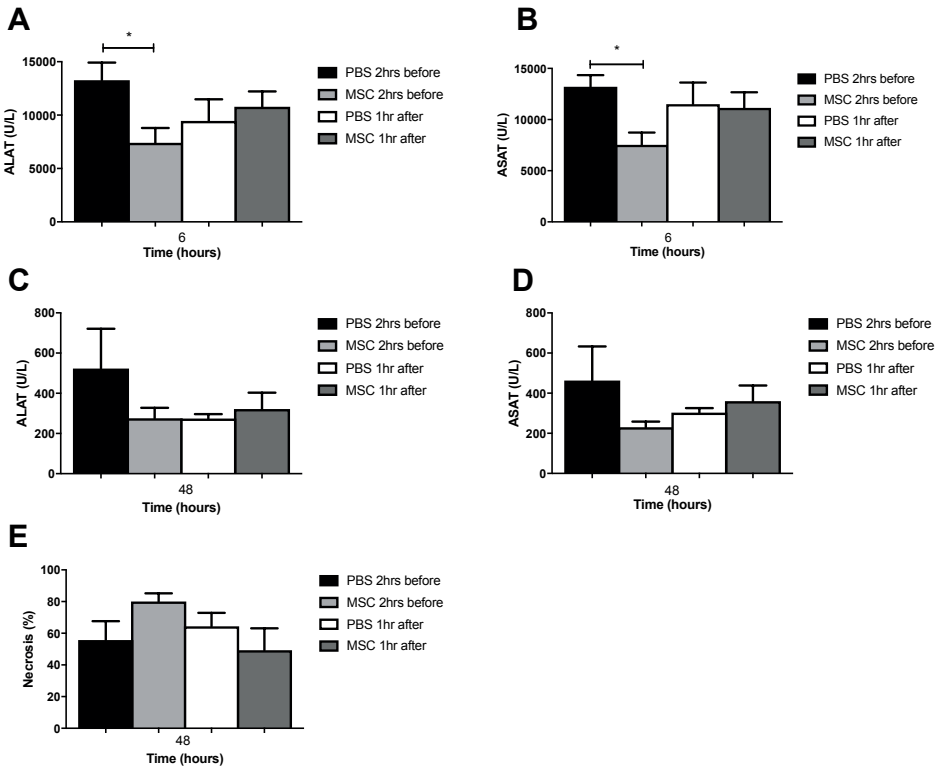


Figure 1. Hepatocellulair injury. **(A)** Six hours after IRI and PH, the serum ALAT concentration was significantly lower in mice treated with MSC 2 hours before hepatic IRI and PH compared to their PBS control. **(B)** Serum ASAT concentration, six hours after reperfusion, was significantly lower in mice treated with MSC 2 hours before IRI and PH compared to their PBS controls. **(C)** Forty-eight hours after IRI and PH there were no significant differences in serum ALAT levels between mice treated with MSC or PBS. **(D)** Serum ASAT concentrations showed no significant differences forty-eight hours after IRI and PH between mice infused with MSC or PBS. **(E)** Forty-eight hours after IRI and PH, livers from mice treated with MSC showed no differences in amount of necrosis compared to their PBS controls. The data are expressed as means ± SEM, ((*P<0.05) vs their PBS controls).

no significant differences in transaminases between mice infused with MSC 2 hours before or 1 hour after reperfusion and PH compared to the mice infused with PBS (**Figure 1C, 1D**). Histological examination of livers 48 hours after reperfusion and PH (**Figure 1E**) revealed no significant differences in amount of hemorrhagic necrosis between mice treated with MSC compared to controls.

Effect of mesenchymal stem cells on inflammation

We investigated the effect of MSC on the inflammation induced by IRI by measuring the expression levels of IL-6 and TNF-α compared with the PBS treated controls. Six hours after reperfusion and PH, there were no significant differences in expression levels of IL-6 between both groups. However, 48 hours after reperfusion expression levels of IL-6 were significantly lower in mice treated with MSC 2 hours before surgery compared to their PBS controls (*P*=0.013). Mice treated with MSC 1 hour after surgery showed no differences (**Figure 2A**). TNF-α did not show significant differences in expression levels after reperfusion and PH in mice infused with MSC or PBS (**Figure 2B**).

In response to hepatic IRI, the cytoprotective gene HO-1 is up-regulated in an attempt to protect the liver against antioxidants. In previous studies, we found that up-regulation

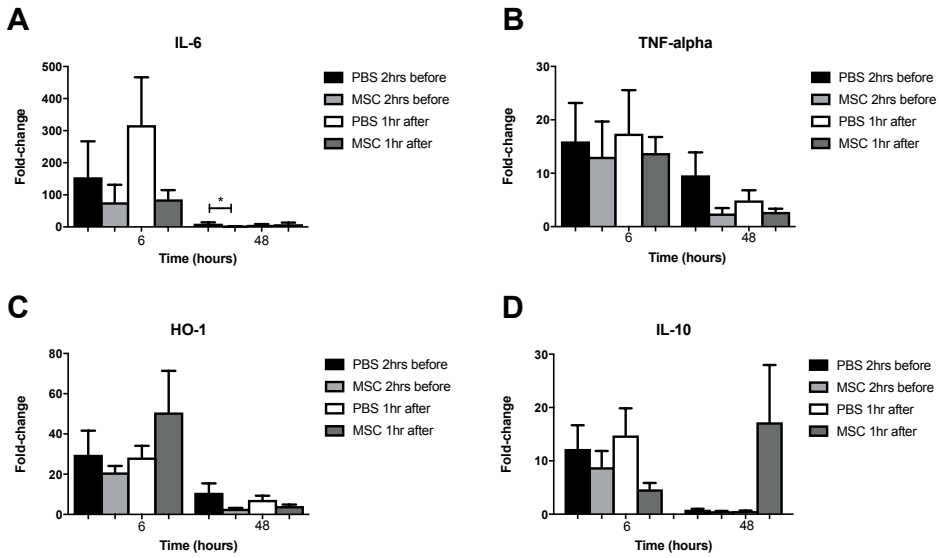


Figure 2. (Anti)-inflammatory- and cytoprotective response. **(A)** mRNA levels of inflammatory marker IL-6 showed no differences six hours after reperfusion. Forty-eight hours after reperfusion mice treated with MSC 2 hours before IRI and PH showed significant lower expression levels of IL-6 compared to PBS treated mice. **(B)** Inflammatory markers TNF-α showed no significant difference after reperfusion. **(C)** Cytoprotective gene HO-1 showed no significant differences between mice treated with MSC or PBS at both time points. **(D)** Anti-inflammatory gene IL-10 showed no significant differences between MSC or PBS treated mice. The data are expressed as means ± SEM, (*P<0.05) vs their PBS controls).

of HO-1 was associated with reduced the liver damage[26]. In the present study, infusion of MSC had no effect on the expression of HO-1 compared to their PBS control group (**Figure 2C**). Anti-inflammatory gene IL-10, showed no differences between mice treated with MSC compared to their PBS controls after both time points (**Figure 2D**).

Effect of mesenchymal stem cells on liver regeneration

The remarkable capacity of the liver to regenerate is important for the postoperative liver function and is influenced by hepatic IRI. We determined liver/body weight ratios 5 days after hepatic IRI and PH of mice infused with MSC 2 hours before- or 1 hour after hepatic IRI and PH, compared with their PBS controls. There were no significant differences in liver weight/total body weight ratios between mice treated with MSC or their PBS controls (**Figure 3A**). Next we investigated hepatocyte proliferation with a PCNA staining (**Figure 3B**).

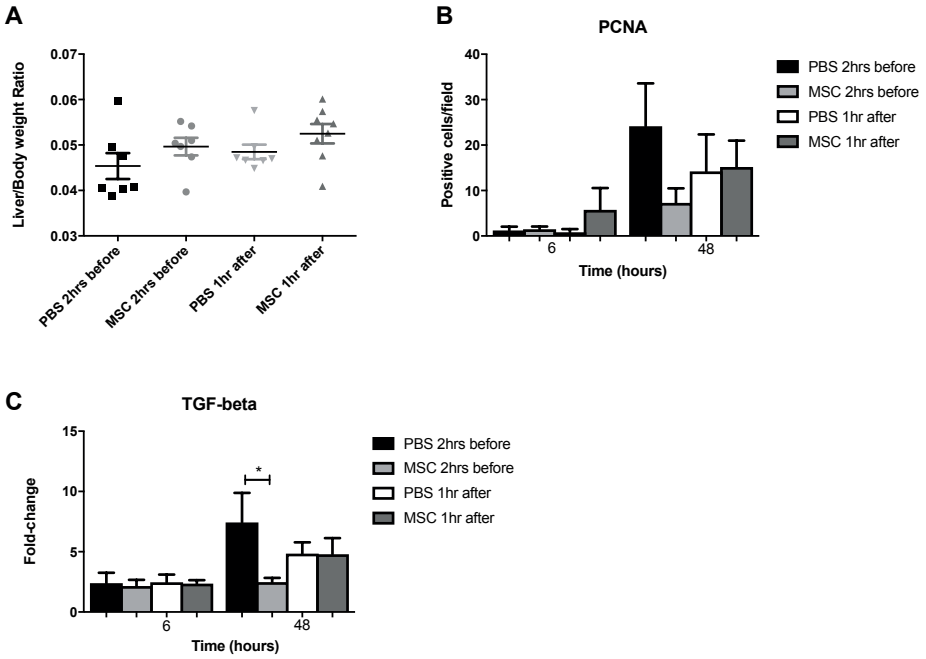


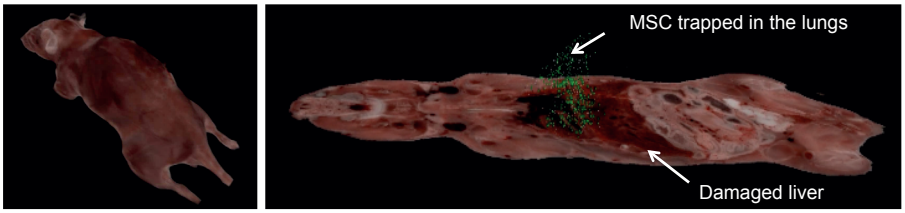
Figure 3. Liver regeneration. **(A)** Liver/body weight ratio 5 days after IRI and PH showed no significant differences between MSC or PBS treated mice. **(B)** After 6 and 48 hours after IRI and PH the numbers of PCNA positive cells were not significantly different. **(C)** Regeneration marker TGF-β showed significantly higher expression levels forty-eight hours after reperfusion in mice treated with PBS 2 hours before IRI and PH. The data are expressed as means ± SEM, ((*P<0.05) vs their PBS controls).

Six hours after hepatic IRI and PH, there was no significant difference between mice infused with MSC or PBS. Forty-eight hours after IRI and PH, the PCNA index peaked but there were no significant differences between the MSC and the PBS groups. Transforming growth factor beta (TGF-β) is a protein involved in the termination response of liver regeneration[27]. Six hours after IRI and PH, there were no differences between the MSC infused mice and their PBS controls. Forty-eight hours after IRI and PH expression levels of TGF-β were significantly lower in mice infused with MSC 2 hours before surgery compared to their PBS controls (P=0.025), mice infused with MSC 1 hour after surgery showed no significant differences (**Figure 3C**).

Fate of intravenously infused mesenchymal stem cells

Using CryoVize whole body imaging, MSC can be detected at single cell level and quantified. Luk et al. (unpublished data) showed by CryoViz imaging that intravenously infused MSC disappear for over 90% within 24 hours after infusion in a healthy mouse but also in a mouse with renal IRI and did not migrate to the damaged organ. We infused 2x10^5 labeled MSC directly after the induction of hepatic IRI. Two hours after IRI the mouse was sacrificed and the MSC were traced. By far, most cells were found in the lungs (45 584 cells), in the damaged liver 3693 cells were present. A few cells were found in other organs and 1218 cells were found in the rest of the mouse. These data show that MSC infused intravenously 2 hours before hepatic IRI are trapped in the lungs, and do not migrate to the injured liver (**Figure 4**). Importantly, 2 hours after infusion of 2x10^5 MSC only 50 607 MSC were found, indicating that many of the infused cells disappeared within 2 hours.

A



B

Specimen	Injected after IRI	Liver	Lung	Spleen	Left kidney	Right kidney	Rest of mouse
2 hours after IRI	200 000	3694 (1.8%)	45 584 (22.8%)	64 (0.03%)	18 (0.009%)	29 (0.01%)	1 218 (0.6%)

Figure 4. In vivo cryo imaging of labeled MSC. **(A)** MSC labeled with Qtracker605, which stains live cells, infused directly after-, and imaged 2 hours after hepatic IRI are present in the lungs and not in the damaged liver. **(B)** Number of detected MSC 2 hours after hepatic IRI, many of the live MSC are trapped in the lungs, but the majority of infused cells cannot be detected, suggesting that these cells are dead.

Discussion

Combined IRI and inadequate tissue regeneration are major causes of morbidity and mortality after LTx and liver resection[12,13,28]. Animal models showed beneficial effects of MSC therapy on repair of injured organs and are able to ameliorate inflammatory processes[13,29-31]. However, the role of MSC therapy in a clinically relevant model of combined hepatic IRI and partial liver resection remains to be established.

In the present study we demonstrated that infusion of $2x10^5$ MSC 2 hours before hepatic IRI and PH significantly decreased ASAT and ALAT levels compared to their PBS controls six hours after IRI and PH, but did not decrease the amount of IRI induced hemorrhagic necrosis. In addition, MSC infused before or after hepatic IRI and PH showed no beneficial effect on liver damage or regeneration. Although the kinetics of markers of injury and inflammation after IRI, and proliferation and termination after resection are relatively well known, their behavior following combined IRI and resection is less clear[32,33].

Serum transaminase levels were significantly lower at 6 hours after IRI and PH in mice that were infused with MSC 2 hours before surgery. Intra-hepatic mRNA IL-6 and TNF-α levels did not provide consistent results. Cytoprotective gene HO-1 showed a trend towards higher levels of expression in mice treated with MSC 1 hour after surgery, whereas IL-10 did not. Despite the significant differences in transaminases between MSC and PBS treated mice, the amount of liver necrosis at 48 hours after surgery, which can be considered the gold standard to measure hepatocellular injury, did not differ between PBS treated controls and any of the MSC treated groups. These data show that MSC infusion may have modulated the early inflammatory response, but had no effect on IRI induced liver damage.

Liver regeneration as assessed by liver/body weight ratio was not significantly different between groups, although there was a trend towards a higher ratio in the MSC treated animals. However, this is not supported by numbers of proliferating cells and TGF-β expression at 48 hours after IRI. However, 48 hours after IRI and PH, TGF-β expression attained significant higher levels in the PBS infused mice before IRI and PH compared to MSC treated mice. On the one hand, this might be explained by the role of TGF-β as a key mediator of tissue fibrosis[34-36]. Rats treated with hepatocyte growth factor (HGF)–expressing MSC after small-for-size liver transplantation showed a decrease in hepatic fibrosis compared to rats treated with PBS[37]. In this model, a suppressed production of TGF-β is correlated with decreased fibrosis. Suggesting that the balance between TGF-β and HGF plays a critical role in the recovery or fibrogenesis of the injured tissues. On the other hand, higher levels of proliferation and TGF-β in control mice in our study may also suggest a more rapid regenerative response in the absence of MSC.

Our findings are in contradiction with the results of Saidi et al.[23]. Using a mouse model with 60 minutes of partial IRI with 70% hepatectomy, mice were treated with 1-2 million human adipose-derived MSC that were administered intravenously 30 minutes before ischemia. Mice treated with MSC showed improved survival, lower levels of ALAT and serum IL-6, and increased numbers of PCNA positive cells. Labelled MSC were localised in the liver for 9 days.

How MSC exert their effect is still matter of debate. Using 3D cryo-imaging, we were able to show that intravenously infused MSC are exclusively trapped in the lungs, do not migrate to the injured liver, and that many infused cells have disappeared from the body 2 hours after infusion. These data support recent findings that infused MSC do not migrate to sites of injury, but are trapped in microcapillary networks, have a short lifespan, and exert their action via paracrine mechanisms[15,19,38-41]. MSC have been observed in liver, spleen[23,42,43] and injured organs[26,40,42] after intravenous infusion. The viability of these cells is not well known, and it has been suggested that they may represent dead or phagocytosed MSC. Despite the hypothesis that the detected MSC are dead, a variety of animal models showed beneficial effects of MSC[13,23,28,43,44] lending further support to the notion that delivery of MSC to the injured organ is not required for their beneficial effects[16,41].

In our study robust beneficial effects of MSC on liver injury and regeneration are lacking while in other studies they have been shown to be effective in ameliorating hepatic IRI and promote liver regeneration[20,23,27,28,43,44]. The variable outcomes in animal studies have many reasons. The number of MSC is a critical factor, yet differs between studies, with reported numbers between 1-3 million MSC in rats and 0.5-2 million MSC in mice[13,22,23,38,45]. We used $2x10^5$ MSC since higher numbers of cells induced liver damage (**Figure S1**). The route of administration, which can be local via the portal vein[13], or systemic via the tail vein,[21] might play an important role in the outcome after MSC infusion. Since it is now known that MSC do not migrate beyond the lungs after intravenous infusion, the systemic route may not be the best route for MSC administration. MSC delivered via the portal vein were detected in the liver until post-operative day 7. However, the effect on liver damage and regeneration was comparable to infusion of MSC via the tail vein[13]. Also, the timing of MSC administration might play an important role in the outcome after IRI and PH.

Intravenous infusion of $2x10^5$ MSC 2 hours before- or 1 hour after partial IRI and 30% PH did not ameliorate liver damage or improve regeneration. Using 3D cryo-imaging, we showed that many of the infused MSC disappear within 2 hours and that the remaining cells are trapped in the lungs and do not migrate towards the damaged liver. More research is needed to the sources of these inconsistent results to improve the reliability of MSC therapy and get closer to translation to the clinical arena.

References

1. Shiffman ML, Brown RS, Jr., Olthoff KM, et al. Living donor liver transplantation: summary of a conference at The National Institutes of Health. Liver Transpl 2002;8:174-88.
2. Karoui M, Penna C, Amin-Hashem M, et al. Influence of preoperative chemotherapy on the risk of major hepatectomy for colorectal liver metastases. Ann Surg 2006;243:1-7.
3. Skullman S, Ihse I, Larsson J. Influence of malnutrition on regeneration and composition of the liver in rats. Acta Chir Scand 1990;156:717-22.
4. Timchenko NA. Aging and liver regeneration. Trends Endocrinol Metab 2009;20:171-6.
5. Schindl MJ, Redhead DN, Fearon KC, et al. The value of residual liver volume as a predictor of hepatic dysfunction and infection after major liver resection. Gut 2005;54:289-96.
6. Zhou Q, Li L, Li J. Stem cells with decellularized liver scaffolds in liver regeneration and their potential clinical applications. Liver Int 2014.
7. Ansari D, Bergenfeldt M, Tingstedt B, Andersson R. Multimodal management of colorectal liver metastases and the effect on regeneration and outcome after liver resection. Scand J Gastroenterol 2012;47:1460-6.
8. Attwa MH, El-Etreby SA. Guide for diagnosis and treatment of hepatocellular carcinoma. World J Hepatol 2015;7:1632-51.
9. Waghray A, Murali AR, Menon KN. Hepatocellular carcinoma: From diagnosis to treatment. World J Hepatol 2015;7:1020-9.
10. Karatzas T, Neri AA, Baibaki ME, Dontas IA. Rodent models of hepatic ischemia-reperfusion injury: time and percentage-related pathophysiological mechanisms. J Surg Res 2014.
11. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. World J Gastroenterol 2013;19:1683-98.
12. Dahm F, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. Am J Transplant 2005;5:2605-10.
13. Kanazawa H, Fujimoto Y, Teratani T, et al. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. PLoS One 2011;6:e19195.
14. Ben-Haim M, Emre S, Fishbein TM, et al. Critical graft size in adult-to-adult living donor liver transplantation: impact of the recipient's disease. Liver Transpl 2001;7:948-53.
15. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. Stem Cells Dev 2013;22:2825-35.
16. Lee JW, Fang X, Krasnodembskaya A, Howard JP, Matthay MA. Concise review: Mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. Stem Cells 2011;29:913-9.
17. Jin G, Qiu G, Wu D, et al. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. Int J Mol Med 2013;31:1395-401.
18. Sun CK, Chang CL, Lin YC, et al. Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats. Crit Care Med 2012;40:1279-90.
19. Seki T, Yokoyama Y, Nagasaki H, Kokuryo T, Nagino M. Adipose tissue-derived mesenchymal stem cell transplantation promotes hepatic regeneration after hepatic ischemia-reperfusion and subsequent hepatectomy in rats. J Surg Res 2012;178:63-70.
20. Kaibori M, Adachi Y, Shimo T, et al. Stimulation of liver regeneration after hepatectomy in mice by injection of bone marrow mesenchymal stem cells via the portal vein. Transplant Proc 2012;44:1107-9.

21. Saidi RF, Rajeshkumar B, Shariftabrizi A, Dresser K, Walter O. Human C1 inhibitor attenuates liver ischemia-reperfusion injury and promotes liver regeneration. J Surg Res 2014;187:660-6.
22. Wang W, Du Z, Yan J, et al. Mesenchymal stem cells promote liver regeneration and prolong survival in small-for-size liver grafts: involvement of C-Jun N-terminal kinase, cyclin D1, and NF-kappaB. PLoS One 2014;9:e112532.
23. Saidi RF, Rajeshkumar B, Shariftabrizi A, et al. Human adipose-derived mesenchymal stem cells attenuate liver ischemia-reperfusion injury and promote liver regeneration. Surgery 2014;156:1225-31.
24. Behr L, Hekmati M, Lucchini A, et al. Evaluation of the effect of autologous mesenchymal stem cell injection in a large-animal model of bilateral kidney ischaemia reperfusion injury. Cell Prolif 2009;42:284-97.
25. Behr L, Hekmati M, Fromont G, et al. Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 2007;107:p65-76.
26. Verweij M, van Ginhoven TM, Mitchell JR, et al. Preoperative fasting protects mice against hepatic ischemia/reperfusion injury: mechanisms and effects on liver regeneration. Liver Transpl 2011;17:695-704.
27. Zimmermann A. Regulation of liver regeneration. Nephrol Dial Transplant 2004;19 Suppl 4:iv6-10.
28. Chen XB, Xu MQ. Primary graft dysfunction after liver transplantation. Hepatobiliary Pancreat Dis Int 2014;13:125-37.
29. Morigi M, Introna M, Imberti B, et al. Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice. Stem Cells 2008;26:2075-82.
30. Aurich H, Sgodda M, Kaltwasser P, et al. Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. Gut 2009;58:570-81.
31. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-89.
32. Elias-Miro M, Jimenez-Castro MB, Rodes J, Peralta C. Current knowledge on oxidative stress in hepatic ischemia/reperfusion. Free Radic Res 2013;47:555-68.
33. van Golen RF, Reiniers MJ, Olthof PB, van Gulik TM, Heger M. Sterile inflammation in hepatic ischemia/reperfusion injury: present concepts and potential therapeutics. J Gastroenterol Hepatol 2013;28:394-400.
34. Border WA, Noble NA. TGF-beta in kidney fibrosis: a target for gene therapy. Kidney Int 1997;51:1388-96.
35. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. J Am Soc Nephrol 2002;13:2600-10.
36. Schnaper HW, Hayashida T, Hubchak SC, Poncelet AC. TGF-beta signal transduction and mesangial cell fibrogenesis. Am J Physiol Renal Physiol 2003;284:F243-52.
37. Yu Y, Lu L, Qian X, et al. Antifibrotic effect of hepatocyte growth factor-expressing mesenchymal stem cells in small-for-size liver transplant rats. Stem Cells Dev 2010;19:903-14.
38. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. Front Immunol 2012;3:297.
39. Barbash IM, Chouraqui P, Baron J, et al. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 2003;108:863-8.
40. Assis AC, Carvalho JL, Jacoby BA, et al. Time-dependent migration of systemically delivered bone marrow mesenchymal stem cells to the infarcted heart. Cell Transplant 2010;19:219-30.

41. Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev 2009;18:683-92.
42. Chapel A, Bertho JM, Bensidhoum M, et al. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 2003;5:1028-38.
43. Nowacki M, Nazarewski L, Pokrywczynska M, et al. Long-term influence of bone marrow-derived mesenchymal stem cells on liver ischemia-reperfusion injury in a rat model. Ann Transplant 2015;20:132-40.
44. Pan GZ, Yang Y, Zhang J, et al. Bone marrow mesenchymal stem cells ameliorate hepatic ischemia/reperfusion injuries via inactivation of the MEK/ERK signaling pathway in rats. J Surg Res 2012;178:935-48.
45. Fu J, Zhang H, Zhuang Y, et al. The role of N-acetyltransferase 8 in mesenchymal stem cell-based therapy for liver ischemia/reperfusion injury in rats. PLoS One 2014;9:e103355.

Supplementary data

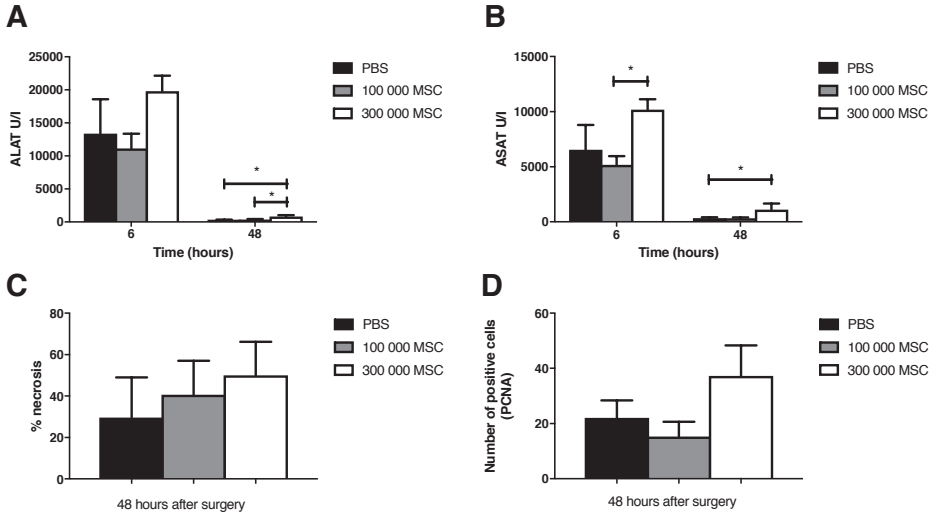
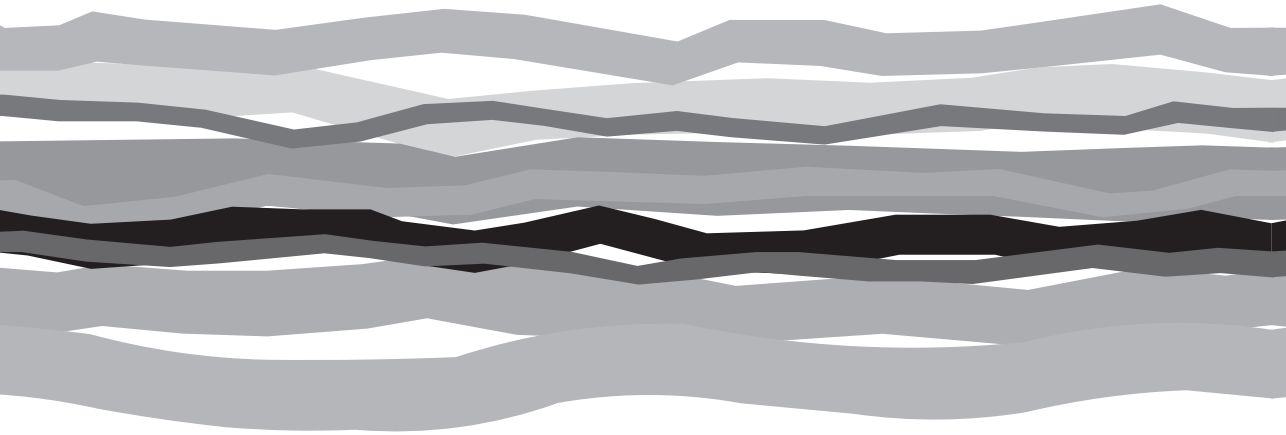
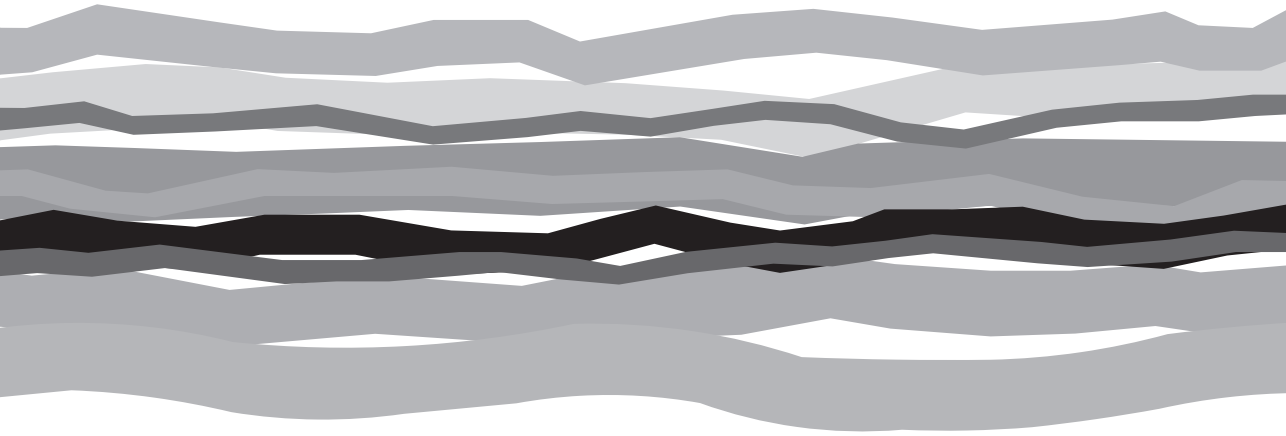


Figure S1. Hepatocellular injury after infusion of PBS, $1x10^5$ or $3x10^5$ MSC directly after IRI and PH. **(A**) Six hours after IRI and PH serum ALAT levels showed no significant differences between PBS, $1x10^5$ or $3x10^5$ MSC infused mice. Forty-eight hours after IRI and PH, mice infused with $3x10^5$ MSC showed significant higher serum levels of ALAT compared to the PBS and $1x10^5$ MSC treated mice. **(B)** Mice infused with $3x10^5$ MSC showed significant higher serum ASAT levels six hours after IRI and PH compared with mice infused with $1x10^5$ MSC. Forty-eight hours after IRI and PH mice treated with $3x10^5$ MSC showed significant higher levels of ASAT compared to PBS treated mice. **(C)** Forty-eight hours after IRI and PH there was no difference in percentage of necrosis in liver tissue between mice infused with PBS, $1x10^5$ or $3x10^5$ MSC. However, mice infused with $3x10^5$ MSC show a trend towards higher percentage of necrosis compared to PBS treated mice. **(D)** The number of PCNA positive cells was not significantly different. However, mice treated with $3x10^5$ MSC might show a trend towards higher number of proliferating cells compared to PBS treated mice. These data suggest that there is more liver damage in mice treated with $3x10^5$ MSC, and hence a stronger regenerative response. The data are expressed as means ± SEM, ((*$P<0.05$) vs PBS or $1x10^5$ MSC infused mice).



Chapter 8

Preoperative fasting protects aneurysmal Fibulin-4 mice against chronic renal damage induced by ischemia reperfusion injury



T.C. Saat, I. van der Pluijm, R.Y. Ridwan, S. van Damme-van den Engel,
P.M. van Heijningen, M.C. Clahsen-van Groningen, H.J.M. Verhagen,
J.N.M. IJzermans, J. Essers, R.W.F de Bruin

Abstract

Aortic aneurysm formation is associated with defective extracellular matrix remodeling, in which Fibulin-4 plays an important role. During the open repair of pararenal aortic aneurysms, renal ischemia reperfusion injury (IRI) is inevitable and causes acute kidney injury and morbidity. Preoperative fasting is a powerful way to increase resistance against IRI. Therefore we investigated the effect of fasting on IRI in heterozygous Fibulin-4 (Fibulin-$4^{+/R}$) mice, which are predisposed to develop aortic aneurysms.

Wild type (WT) and Fibulin-$4^{+/R}$ mice were fed ad libitum or were fasted for 2 days before the induction of renal IRI. Six hours, one day or six months postoperatively serum urea levels, renal histology, matrix metalloproteinase activity and mRNA expression levels of inflammatory and injury genes were used to determine kidney function and damage.

Two days of fasting improved survival the first week after renal IRI in WT mice compared to ad libitum fed mice. Remarkably, AL fed Fibulin-$4^{+/R}$ mice already showed improved survival and kidney function compared to AL fed WT mice, which could not be further improved by fasting. However, six months after renal IRI fasted WT and Fibulin-$4^{+/R}$ mice showed improved survival, kidney function and morphology compared to AL fed mice. Moreover, fasted Fibulin-$4^{+/R}$ mice showed reduced apoptotic cells in the kidney together with increased expression levels of matrix metalloprotease activity, indicative of increased matrix remodeling in the kidney.

Mice predisposed to developing aortic aneurysms are protected against the short-term-, but not long-term consequences of renal IRI. Preoperative fasting protects against renal IRI and prevents deterioration of kidney function and morphology. These data suggest that preoperative fasting may decrease renal damage in patients undergoing open abdominal aneurysm repair.

Introduction

Pararenal aortic aneurysms (PRAAs) are characterized by the absence of normal aorta structure between the upper extent of the aneurysm and the renal arteries or extent to the renal arteries, and cover 8-20% of the abdominal aortic aneurysms (AAAs)[1,2]. Standard endovascular aneurysm repair (EVAR) is feasible in most cases of AAAs and reduces morbidity and mortality[3]. However, the feasibility of standard EVAR in patients with PRAAs is limited by strict anatomical requirements, high costs, and lengthy manufacturing lead-times[2,3]. Therefore, open repair of PRAAs is still used on one in five patients[4,5].

The development of aortic aneurysms is associated with extrinsic or intrinsic defects of the blood vessel wall[6]. AAAs show destructive extracellular matrix (ECM) remodeling, apoptosis of smooth muscle cells, and inflammatory cell infiltration. Besides gender, age, and lifestyle-related risk factors, there is evidence that genetic determinants play a role in the development of AAAs[7]. Fibulin-4 is an elastic fiber-associated glycoprotein involved in stabilizing the organization of ECM structures and plays a role in elasticity and integrity[8-10]. Mice without expression of Fibulin-4 (Fibulin-$4^{-/-}$) display embryonic death due to aortic rupture[8,9]. In a Fibulin-4 knockdown mouse model, mice homozygous for the Fibulin-4 reduced expression allele (Fibulin-$4^{R/R}$) have a 4-fold reduced Fibulin-4 expression. These mice show disorganized elastic fiber networks and may die suddenly of cardiovascular complications before reaching weaning age[8,11]. Heterozygous mice (Fibulin-$4^{+/R}$) have a 2-fold reduced Fibulin-4 expression, which results in clear aberrations in elastin formation with occasionally small aneurysm formation and may therefore provide invaluable information regarding the early onset of aortic degeneration and the effect of stressors on blood vessel walls

Renal ischemia-reperfusion injury (IRI) is an inevitable consequence of open surgical PRAA repair. Ischemia is caused by interruption of the renal blood flow and is associated with a drop in oxygen and nutrient concentration, which causes accumulation of metabolic waste products[12]. Reperfusion of the kidney activates an inflammatory response, triggers apoptotic cell death and results in reactive oxygen species formation[13]. IRI is a risk factor for acute kidney injury that can lead to dialysis and is associated with prolonged hospitalization and risk to develop chronic deterioration of kidney function[14]. Previous studies in mice showed that preoperative fasting protects against renal IRI[15,16]. Here, we used the Fibulin-$4^{+/R}$ mouse to explore the effect of oxidative stress induced by IRI on kidney damage directly after surgery and in the long-term. In addition, we studied the effect of preoperative fasting in Fibulin-$4^{+/R}$ mice after inducing renal IRI on survival, kidney function and morphology.

Material and methods

Mouse model

Heterozygous Fibulin-4 mutant mice (Fibulin-$4^{+/R}$) were generated as described previously[11]. Male wild type and Fibulin-$4^{+/R}$ mice aged 8-10 weeks were used for the experiments. The mice were housed in individually ventilated cages (2-3 animals/cage) under standard conditions. Mice were earmarked and genotyped 4 weeks after birth to avoid stress-related vascular injury. Animals were housed at the Animal Resource Centre (Erasmus University Medical Centre), which operates in compliance with the "Animal Welfare Act" of the Dutch government, using the "Guide for the Care and Use of Laboratory Animals" as its standard. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. An independent Animal Ethics Committee of the Erasmus Medical Center (Stichting DEC Consult) approved these studies (permit number 105-12-16), in accordance with national and international guidelines.

Experimental design

At the start of the experiment, wild type (WT) mice and heterozygous Fibulin-4 mutant (Fibulin-$4^{+/R}$) mice were randomly divided into two different groups, ad libitum (AL) fed or 2 days of fasting. All mice were transferred to clean cages at 4:00 pm. The AL fed mice had free access to food and water. Two days before surgery, mice in the fasted groups did not receive any food but had AL access to water. Despite significant weight loss as a result of fasting, no morbidity or mortality was observed as a function of fasting alone. Mouse body weight as well as food intake was recorded daily. Following induction of renal IRI, mice with symptoms indicative of irreversible acute kidney injury were sacrificed. AL fed and fasted mice were euthanized before surgery (3-5 per group), six hours (4-6 per group), 24 hours (3-7 per group) 7 days (8-12 per group) and 6 months (5-7 per group) after renal IRI mice were sacrificed to collect blood, and kidney tissue (**Table S1A**). At 6 months the aorta was dissected from the heart until the bifurcation for further analysis (**Table S1B**).

Renal ischemia reperfusion injury

Surgeries were performed between 9:00 am and 1:00 pm. Mice were anesthetized by isoflurane inhalation (5% isoflurane initially and then 2-2.5% with oxygen for maintenance), and placed on heating pads of 37°C to maintain body temperature until recovery from anesthesia. Following a midline abdominal incision, the left and right renal pedicles were localized and dissected from the surrounding fat, and a non-traumatic micro-vascular clamp was placed across the vessels. After inspection for signs of ischemia (purple coloration of kidneys), the incision was covered with a phosphate buffered

saline (PBS) soaked cotton and aluminum foil to maintain body temperature. In the short-term experiments, kidneys underwent 37 minutes of ischemia. In previous studies[15] we consistently found mortality of approximately 80% due to acute kidney failure following 37 min warm ischemia, making it difficult to study the long-term consequences of IRI. To circumvent this problem, in the long-term experiments, kidneys underwent 30 minutes of ischemia. With this amount of damage survival rates are higher, allowing following these animals beyond the first postoperative days. After clamp removal, the abdomen was closed in two layers using Vicryl 4-0 sutures. All mice received 0.5 mL PBS subcutaneously immediately after surgery for the fluid balance. Mice were placed under a heating lamp until recovered from anesthesia. Directly after surgery all animals had AL access to food and water[15].

Kidney function

Kidney function, as assessed by serum urea concentrations, was determined using the Quantichromä Urea Assay Kit (DIUR-500) (BioAssay Systems, Hayward, USA) following the manufactures instructions.

Histopathological analysis

Kidneys were harvested, fixed for 24 hours in formalin and embedded in paraffin. Three-micrometer thick sections were cut and stained with periodic acid Schiff (PAS) staining. The percentage of damaged tubules in the corticomedullary junction was scored using a five-point scale according the following criteria: tubular dilatation, cast deposition, brush border loss, and necrosis in 10 randomly chosen, non-overlapping fields with x400 magnification. Lesions were scaled from 0 to 5: 0 is normal, 1 is mild, 2 is moderate, 3 is severe, 4 very severe and 5 is extensive damage[17].

In kidneys harvested six months after renal IRI an additional Sirius Red staining was performed to determine the amount of interstitial fibrosis. A renal pathologist (MCG) blinded for genotype and intervention used the Banff classification[18] to determine interstitial fibrosis in the cortex: 0 (< 5% fibrosis), 1 (6-25% fibrosis), 2 (26-50% fibrosis) and 3 (>50% fibrosis).

TUNEL staining

Kidney specimens were fixed overnight in 10% buffered formalin, paraffin-embedded, sectioned at 5 micrometer, and mounted on Superfrost Plus slides. Paraffin sections were employed for TdT-mediated dUTP Nick-End Labeling (TUNEL) assay using a commercial kit (Apoptag Plus Peroxidase in situ apoptosis detection kit, Millipore). Sections were deparaffinized and incubated as described by the manufacturer. Kidney sections were scanned with the Nanozoomer-XR (Hamamatsu Photonics, NanoZoomer-XR), after

which TUNEL positive cells were counted in 3 slides per animal, and corrected for kidney area.

Molecular imaging for MMP activation

We used vascular fluorescent mediated tomography (FMT) imaging with near-infrared fluorescent protease activatable probes as previously described[8,19]. FMT imaging was performed using an FMT 2500 system (Perkin Elmer, Inc.) at 680/700 nm excitation and emission wavelengths, 24 hours after tail vein injection of 2 nmol per 25 g bodyweight of the MMPSense^{TM}680 near-infrared fluorescent probe (Perkin Elmer, Inc.), mice were imaged in a portable animal imaging cassette between optically translucent windows. The FMT 2500 quantitative tomography software was then used to calculate 3D fluorochrome concentration distribution of the fluorescent signal. After fluorescence imaging, kidneys were harvested and fluorescence was quantified using the FMT 2500 or Odyssey imaging systems (LI-COR Inc.). Near-infrared images were obtained in the 700-nm channel.

Quantitative RT-PCR

Total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), followed by a DNase treatment (RQ1 RNase-Free DNase; Promega Benelux B.V., Leiden, the Netherlands), and reverse transcribed to complementary DNA using random hexamer primers and Superscript II RT (both from Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. Quantitative real-time PCR was performed using a MyiQ single-color Real-Time PCR Detection System with SYBR Green incorporation (both from Bio-Rad Laboratories B.V., Veenendaal, the Netherlands; primer sequences are available upon request). As a housekeeping gene we used B2M. Besides that, we measured gene expression levels of inflammatory marker IL-6, cytoprotective gene HO-1 and kidney injury molecule-1 (Kim-1). The Dixons Q test was used to eliminate the outliers. The relative expression was calculated as $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$, corrected for expression levels in healthy wild type control animals. Each sample was tested at least in duplicate.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Differences in groups were analyzed by Mann-Whitney U test. Differences in survival rates were analyzed by Log-rank test using SPSSv21. Differences were considered significant at P values less≤ 0.05.

Results

Baseline differences in kidney functional parameters between Fibulin-4$^{+/R}$ and WT mice

At baseline, before the induction of renal IRI, serum urea levels were similar between diets and genotypes (**Figure 1A**). Also, overall kidney pathology was similar between the two genotypes. However, tubular injury seemed slightly increased in kidneys of AL fed Fibulin-4$^{+/R}$ mice compared to AL fed WT but did not reached significant differences

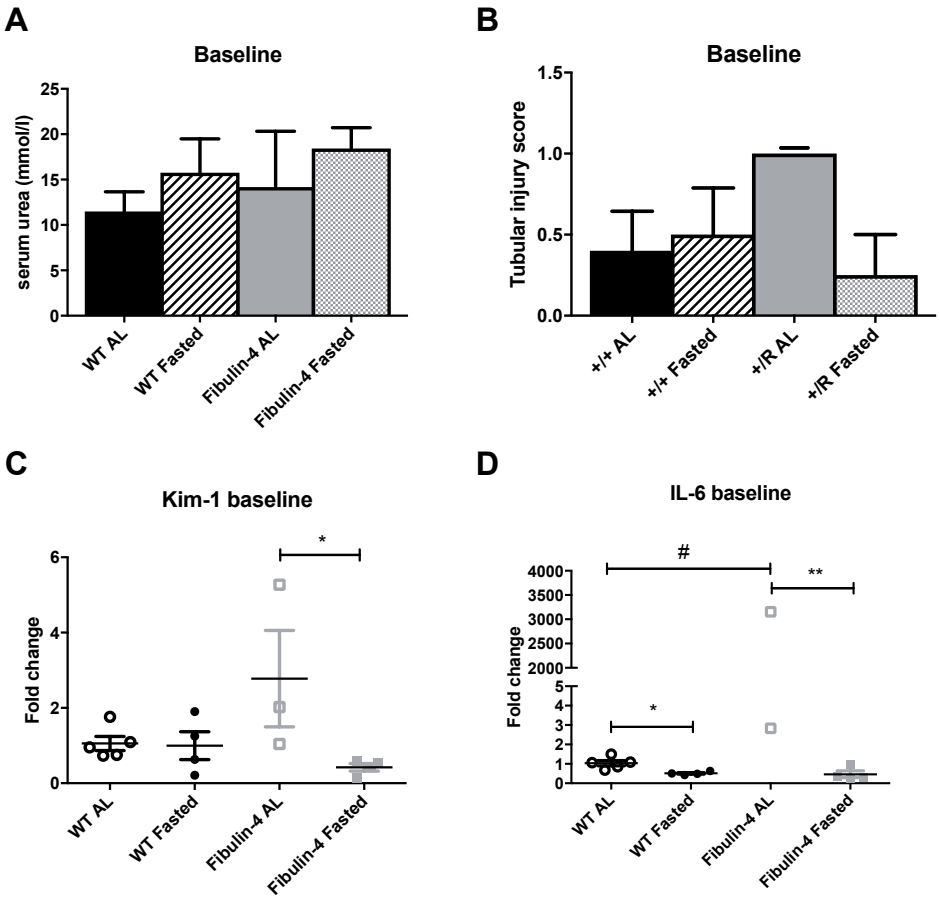


Figure 1. Baseline kidney injury parameters differ between AL-fed Fibulin-4$^{+/R}$ and WT mice. (A) At baseline, serum urea levels showed no differences between genotypes or diets. **(B)** AL-fed Fibulin-4$^{+/R}$ kidneys showed slightly increased tubular injury compared to WT, which was not observed in Fibulin-4$^{+/R}$ mice after fasting. **(C)** At baseline, in AL-fed Fibulin-4$^{+/R}$ mice Kim-1 expression levels were higher compared to AL-fed WT mice, which was significantly reduced in fasted Fibulin-4$^{+/R}$ mice (*p=0.034). **(D)** Inflammatory gene IL-6 is up-regulated in AL-fed Fibulin-4$^{+/R}$ compared WT kidneys (#P=0.025) which was significantly reduced after fasting in both WT (*p=0.014) and Fibulin-4$^{+/R}$ kidneys (**P=0.034).

(**Figure 1B**). As additional measure of renal injury, mRNA expression levels of kidney injury molecule-1 (Kim-1) were used. Already at baseline, Kim-1 levels were increased in AL fed Fibulin-4$^{+/R}$ compared to WT mice, which could be significantly reduced by fasting (**Figure 1C**). Similarly, before induction of renal IRI, mRNA expression levels of inflammatory gene IL-6 seemed increased in AL-fed Fibulin-4$^{+/R}$ mice, which was significantly decreased after fasting in both WT and Fibulin-4$^{+/R}$ mice (**Figure 1D**). Taken together, it seems that at baseline AL fed Fibulin-4$^{+/R}$ mice have a slight increase in kidney injury parameters, which can be significantly reversed by fasting.

AL fed Fibulin-4$^{+/R}$ animals are protected against renal IRI compared to WT controls

During the 2 days of fasting, WT and Fibulin-4$^{+/R}$ mice lost significant weight (**Figure 2A**), while AL fed mice maintained their body weight (WT *p<0.01;* Fibulin-4$^{+/R}$ *p=0.025*). One day after induction of renal IRI, fasted mice showed an increased body weight. Interestingly, while AL fed WT mice had lower body weights compared to AL fed Fibulin-4$^{+/R}$ mice (***p=0.046*). Directly after renal IRI, fasted WT and Fibulin-4$^{+/R}$ mice started eating which resulted in a higher postoperative calorie intake compared to their AL fed controls (**Figure 2B**). After induction of renal IRI, many AL fed WT mice had to be sacrificed due to signs indicative of irreversible kidney injury within seven days (**Figure 2C**). In contrast, 67% of AL fed Fibulin-4 mice survived 7 days after IRI *($p=0.001*). Whereas fasted WT mice showed no signs of morbidity and all survived (***p<0.001 compared to AL fed WT*), fasting did not further increase the survival of Fibulin-4$^{+/R}$ animals. Serum urea levels at 48 hours (**Figure 2D**) were consistent with survival data. Serum urea levels in AL fed WT mice were significantly higher compared to fasted WT mice (**p=0.004*) and AL fed Fibulin-4$^{+/R}$ mice (#*p=0.044*). In agreement with the survival rates, no differences were found in serum urea levels between AL fed or fasted Fibulin-4$^{+/R}$ mice (**Figure 2D**). Tubular injury was determined in kidneys harvested 24 hours after renal IRI. Kidneys from AL fed WT and Fibulin-4$^{+/R}$ mice showed widespread tubular necrosis, tubular dilatation and brush border loss, while fasted WT and Fibulin-4$^{+/R}$ mice showed less tubular injury (*p=0.018* in both cases) (**Figure 2E and F**). In summary, although Fibulin-4$^{+/R}$ mice seem to have somewhat increased kidney injury parameters at baseline, they show increased survival after 37 minutes of renal IRI, which could not be further increased by fasting.

Six hours after renal IRI, Kim-1 expression was strongly increased compared to baseline levels, with a trend towards lower levels in fasted WT and Fibulin-4$^{+/R}$ mice (**Figure 3A**). One day after renal IRI, Kim-1 expression was further increased with no significant difference between AL fed and fasted WT mice. However, AL fed Fibulin-4$^{+/R}$ mice showed significant lower expression levels of Kim-1 compared to AL fed WT mice (*p<0.01*) and fasted Fibulin-4$^{+/R}$ mice (*p<0.01*) (**Figure 3B**). Six hours after renal IRI, IL-6 levels were strongly increased in AL fed WT and Fibulin-4$^{+/R}$ mice, which was significantly reduced

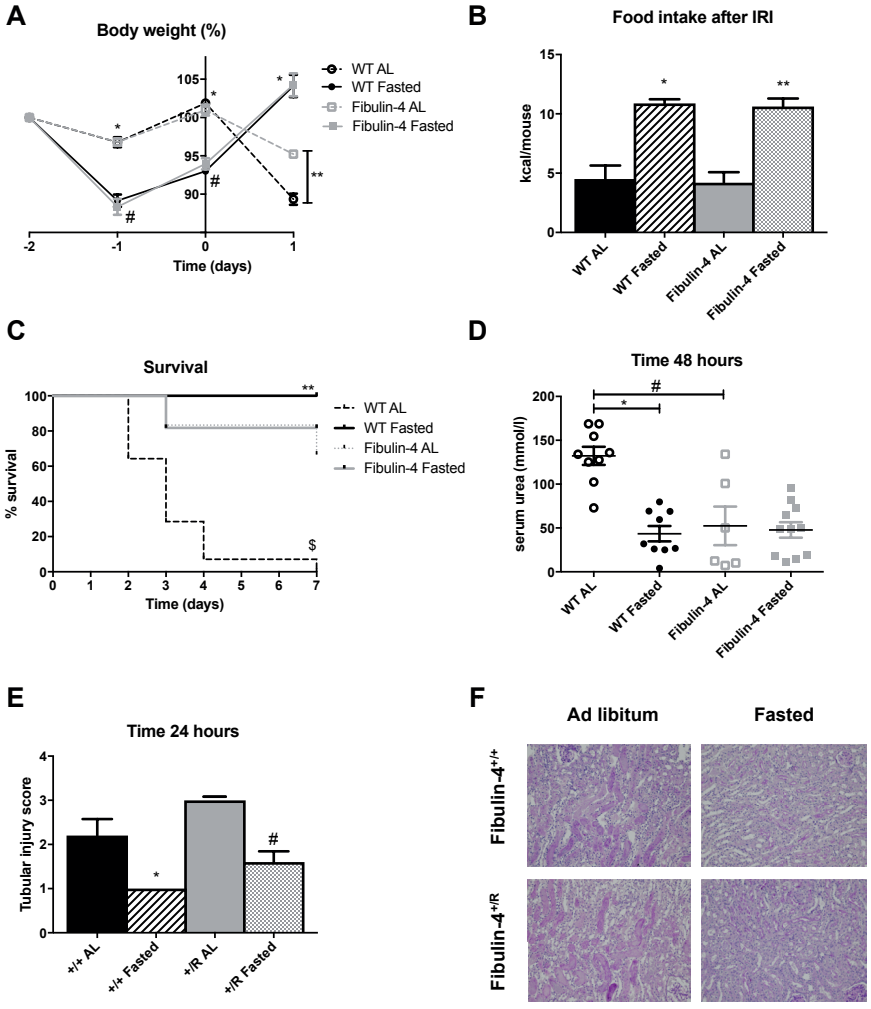


Figure 2. Short-term outcome after 37 minutes IRI in WT and Fibulin-4$^{+/R}$ mice. (A) During the dietary intervention, fasted mice had a significantly lower body weight compared to AL fed WT (*p<0.01) and Fibulin-4$^{+/R}$ mice (#p=0.025). After renal IRI (t=0 in the graph), fasted mice gained weight, while AL fed mice lost weight. At day 1 after IRI, AL fed WT mice had a significantly lower body weight compared to fasted WT (*p<0.01) and AL fed Fibulin-4$^{+/R}$ mice (**p=0.046). **(B)** At postoperative day 1, fasted WT and Fibulin-4$^{+/R}$ mice had a higher calorie intake compared to their AL fed controls (*p=0.004 and **p=0.001, respectively). **(C)** Seven days after the induction of renal ischemia none of the AL fed WT mice survived, whereas AL fed Fibulin-4$^{+/R}$ mice showed a significantly higher survival rate of 66.7% ($p<0.01). One week after renal IRI, all 2-day fasted WT mice survived (**p<0.001 compared to AL-fed WT) and 81.8% of the 2-day fasted Fibulin-4$^{+/R}$ mice survived (not significant compared to AL fed Fibulin-4$^{+/R}$). **(D)** AL fed Fibulin-4$^{+/R}$ mice showed significant lower serum urea levels compared to AL fed WT mice (#p=0.044) 48 hours after IRI. Two days of fasting significantly lowered the serum urea levels in WT mice (*p=0.004), but in the Fibulin-4$^{+/R}$ fasting did not result in further decrease of serum urea levels. **(E, F)** Kidneys from AL fed mice showed widespread tubular necrosis, tubular dilatation and brush border loss, 24 hours after IRI. Fasting significantly reduced tubular injury in both WT and Fibulin-4$^{+/R}$ mice (*p=0.018; #p=0.018).

by fasting in Fibulin-4$^{+/R}$ mice (*$p=0.025$) (**Figure 3C and D**). The levels were returning to baseline 24 hours after IRI.

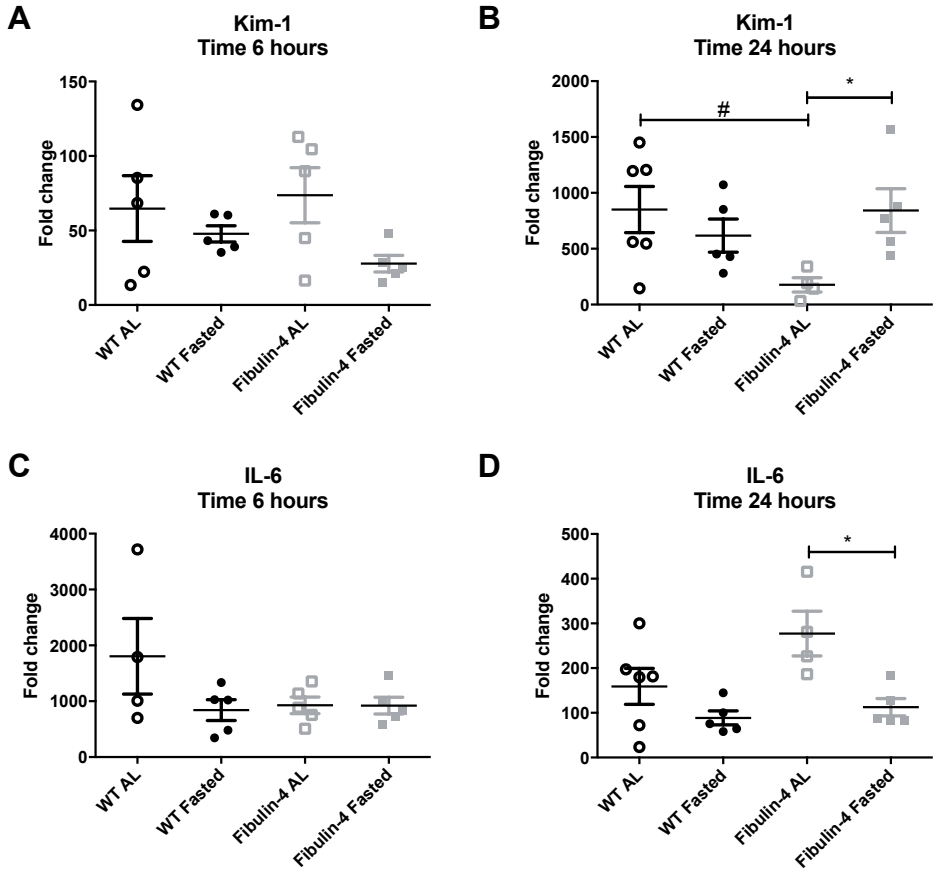


Figure 3. Renal injury and inflammatory response after renal IRI. (A) Six hours after renal IRI, Kim-1 RNA expression levels showed no differences between AL fed and fasted mice. **(B)** AL fed Fibulin-4$^{+/R}$ mice showed significantly lower Kim-1 RNA expression levels compared to AL fed WT mice 24 hours after IRI (#p<0.01). These levels were significantly reduced in 2-day fasted Fibulin-4$^{+/R}$ mice (*p<0.01). **(C)** Six hours after renal IRI there were no significant differences in IL-6 RNA expression levels between the different experimental groups. **(D)** Twenty-four hours after renal IRI AL fed Fibulin-4$^{+/R}$ mice showed an up-regulation of IL-6, which was significantly reduced by fasting (*p=0.025).

Fasting improves long-term survival, postoperative recovery and kidney function in WT and Fibulin-4$^{+/R}$ mice

As we observed differences between WT and Fibulin-4$^{+/R}$ mice in kidney injury parameters and short-term survival after 37 minutes of renal IRI, we wondered how this would affect long-term kidney function. To study these long-term effects, we used a milder model of renal IRI (30 minutes of ischemia), allowing more animals to survive the injury

and to be examined at 6 months of age. During the dietary intervention, similar to the previous experiment, mice lost around 10% of their body weight and had a significantly lower body weight compared to their AL controls (*p<0.01*) (**Figure 4A**). One day after surgery, fasted WT and Fibulin-$4^{+/R}$ mice increased their body weight, while the AL fed WT and Fibulin-$4^{+/R}$ mice lost weight. Also, at postoperative day 1, fasted WT and Fibulin-$4^{+/R}$ mice ate significant more calories compared to the AL fed WT and Fibulin-$4^{+/R}$ mice, which is in correlation with their body weight (*p<0.01*) (**Figure 4B**). Six months after the induction of renal IRI there was no significant difference in body weight between fasted or AL fed WT and Fibulin-$4^{+/R}$ mice (data not shown). Comparable to 37 minutes of renal IRI, at postoperative day 7 more AL fed Fibulin-$4^{+/R}$ were alive than AL fed WT animals (70% versus 60%) (**Figure S1**). After 6 months, all fasted WT and Fibulin-$4^{+/R}$ mice were alive, whereas 60% of the AL fed WT and Fibulin-$4^{+/R}$ mice were deceased (**Figure S1**). Serum urea levels were used as an indicator of kidney function and 48 hours after renal IRI, and showed no significant differences (**Figure 4C**). At 6 months after IRI, fasted WT and Fibulin-$4^{+/R}$ mice showed significantly lower tubular injury score (*p<0.01*) and a reduction in number of apoptotic cells compared to their AL fed controls (**Figure 4D,E,G**). In addition, Sirius red staining showed significantly less fibrosis in the renal cortex of fasted WT and Fibulin-$4^{+/R}$ mice (*p<0.05*) (**Figure 4F,H**), compared to Al fed controls. Interestingly, only 2 days of fasting has significant effects on kidney injury, even after 6 months.

Fasting results in lower MMP expression in Fibulin-$4^{+/R}$ kidneys 6 months after the induction of renal IRI

Microscopically, AL fed WT and Fibulin-$4^{+/R}$ kidneys showed increased tubular injury and interstitial fibrosis (**Figure 4F, Figure 4H**), and macroscopically these kidneys were smaller, dystrophic and some developed renal cysts (data not shown). Since these parameters were significantly reduced after fasting, this seemed a long-term effect of renal IRI. We therefore next examined the potential long-term impact of IRI on matrix remodeling in the kidney, by determining matrix metalloprotease activity (**Figure 5A**).

In control age matched controls, no significant differences between the different genotypes or diets in matrix metalloproteinases activity were found (**Figure 5B**). Kidneys from WT mice did not show any differences between AL fed or fasted mice 6 months after IRI (**Figure 5C**). In fasted Fibulin-$4^{+/R}$ mice, however, MMP activity was significantly higher than in AL fed animals (*p=0.011*). This increase in MMP activity did not correlate to the degree of fibrosis in the kidney as determined in Sirius Red stained sections in **Figure 4E** (data not shown). These data might indicate, that in Fibulin-$4^{+/R}$ kidneys, fasting induces long-term matrix remodeling after renal IRI.

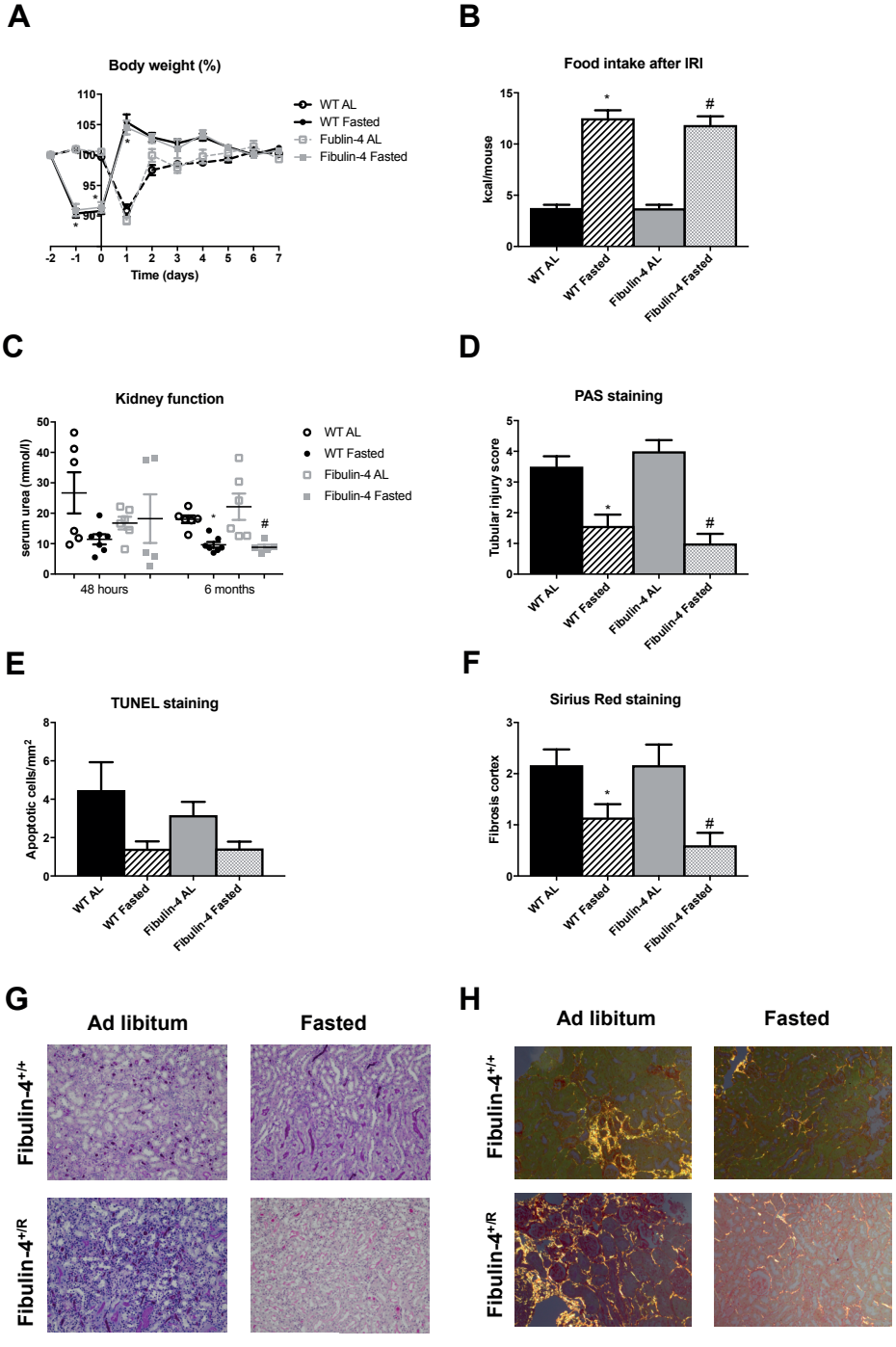


Figure 4. Fasting improves postoperative recovery, kidney function and long-term survival in WT and Fibulin-4$^{+/R}$ mice

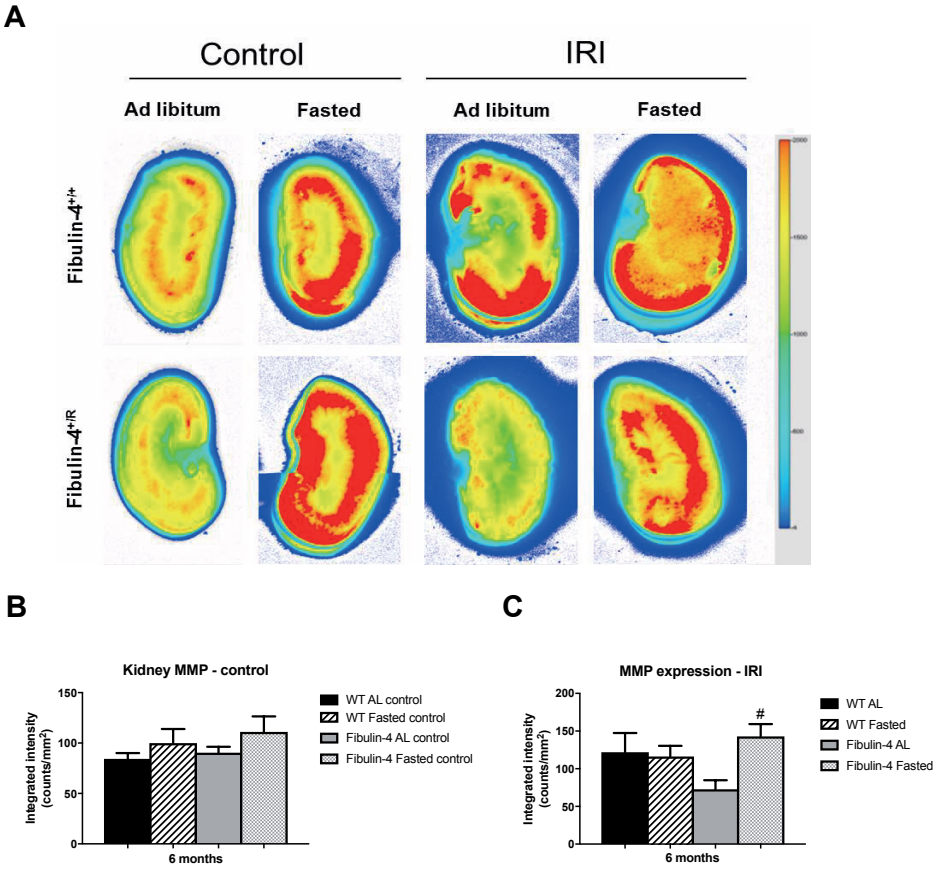


Figure 5. Fasting results in lower MMP expression in Fibulin-4$^{+/R}$ kidneys 6 months after the induction of renal IRI. (A) *Ex vivo* fluorescent images of AL fed and fasted WT and Fibulin-4$^{+/R}$ mice after injection of the MMP probe, 6 months after IRI. **(B)** Control mice showed no significant differences between genotypes, or diet in MMP expression after IRI. **(C)** Six months after renal IRI, WT mice showed no differences in MMP expression levels between AL fed or fasted mice. However, fasted Fibulin-4$^{+/R}$ mice had significant lower levels of MMP compared to AL fed Fibulin-4$^{+/R}$ mice (#p=0.011).

← **Figure 4. Fasting improves postoperative recovery, kidney function and long-term survival in WT and Fibulin-4$^{+/R}$ mice continued (A)** After 2 days of fasting, the body weight of WT and Fibulin-4$^{+/R}$ mice strongly decreased compared to the AL fed mice (*p<0.01). However, after the induction of renal IRI, body weight of the fasted mice increased rapidly, around 13%, whereas the AL fed mice lost 9% of their body weight (*p<0.01). Postoperative day 7 showed no differences between the body weights of fasted or AL fed WT and Fibulin-4$^{+/R}$ mice. **(B)** Directly after surgery, fasted WT and Fibulin-4$^{+/R}$ mice started eating, which resulted in a higher calorie intake compared to their AL fed controls (*p<0.01). **(C)** No significant differences in serum urea levels were observed between fasted and AL fed WT and Fibulin-4$^{+/R}$ mice 48 hours after renal IRI. Remarkably, after 6 months serum urea levels were significantly lower in fasted mice compared to their AL fed controls (*p<0.01). **(D)** Two days of fasting resulted in significantly less tubular injury in both WT and Fibulin-4$^{+/R}$ kidneys compared to their AL fed controls (WT *p<0.01;Fibulin-4$^{+/R}$ #p<0.01). **(E, F)** Similarly, two days of fasting resulted in significantly less interstitial fibrosis in both WT and Fibulin-4$^{+/R}$ kidneys compared to their AL fed controls (*p<0.05; #p<0.05), and less apoptosis. **(G,H)** Representative histological PAS and Sirius Red stained sections from AL fed and fasted WT and Fibulin-4$^{+/R}$ mice.

Discussion

Despite the reduction in morbidity and mortality after the use of standard EVAR, still one in five patients undergo open repair of aneurysms, especially patients with PRAAs[4,5]. During open repair of PRAAs renal IRI is inevitable and a major risk factor for acute kidney injury, which causes morbidity and mortality[14]. Another factor in the development and outcome of aortic aneurysms in patients might be genetic determinants. In the present study, we used the Fibulin-4$^{+/R}$ mouse model with a 2-fold reduced Fibulin-4 expression. These mice match those patients with sporadic and barely detectable forms of aneurysms, which might make them more vulnerable to a worse postoperative outcome. Previous studies showed that preoperative fasting improved survival and kidney function one week after renal IRI[15]. However, the beneficial effects of preoperative fasting on renal IRI in mice prone to develop aneurysms, and in the long-term, are unknown.

Interestingly, before the induction of renal IRI, Fibulin-4$^{+/R}$ mice showed signs of increased kidney injury compared to WT mice. In agreement with previous studies[15,16], our study showed the same beneficial effect of preoperative fasting on renal IRI in WT mice. Fasting reduced injury and inflammation of the kidney and improved survival, kidney function and postoperative recovery compared to AL fed WT mice. In contrast to what we expected, Fibulin-4$^{+/R}$ mice were protected against renal IRI compared to WT mice, and fasting could therefore not further improve outcome following injury on the short-term. These data would imply that the mild kidney injury in Fibulin-4$^{+/R}$ mice induces an increased resistance against oxidative damage. Although counterintuitive, we previously showed that mice harboring a genetic defect in a gene involved in the repair of DNA damage also showed increased resistance against renal IRI[20]. This phenotype has been interpreted as an adaptive response to unrepaired endogenous DNA damage engaged to protect from further oxidative injury[21]. A prediction of this interpretation is that the low grade kidney injury in Fibulin-4$^{+/R}$ mice, most probably due to mild extracellular matrix degeneration observed in these animals[8], induces a similar response which renders them resistant to acute oxidative stress. In the long-term no differences were observed in survival between ad libitum fed WT and Fibulin-4$^{+/R}$ mice, suggesting that the potential survival response is only effective in reducing acute damage. Remarkably, even six months after the induction of renal IRI, 2 days of preoperative fasting proved beneficial in both WT as well as Fibulin-4$^{+/R}$ mice; all fasted mice were alive compared to only 60% of the AL fed mice. Moreover, fasting also prevented kidney injury induced by renal IRI. Fasted WT and Fibulin-4$^{+/R}$ mice showed lower serum urea levels compared to their AL fed controls. Kidneys from AL fed Fibulin-4$^{+/R}$ mice were small, dystrophic and some developed renal cysts, but none of these abnormalities were seen in kidneys from fasted Fibulin-4$^{+/R}$ or WT mice. In addition, kidney sections from fasted mice showed less tubular injury, less apoptosis and less fibrosis compared to AL fed controls.

MMPs are responsible for extracellular matrix remodeling; they directly activate cytokines, chemokines, growth factors and other proteases[22,23]. Previously we showed that in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice MMP expression levels were up-regulated in the aorta even before the aneurysm actually was formed[8]. Therefore, we were curious to determine the expression levels of MMP in the kidneys six months after renal IRI. The AL fed or fasted WT mice did not show any differences in MMP expression levels. In contrast, fasted Fibulin-$4^{+/R}$ mice had increased MMP expression levels compared to AL fed Fibulin-$4^{+/R}$ mice. MMPs have been shown to be involved in acute and chronic renal injury and might play a role in tubular atrophy and fibrosis[24]. Besides that, after renal IRI MMP expression levels increased suggesting that MMPs may regulate the inflammatory response[25]. In a MMP-2-deficient mouse model the degree of tubular injury, necrosis, apoptosis and renal dysfunction was markedly less compared to wild type mice[22]. Therefore, inhibition of MMPs might protect against kidney injury, which has created interest in MMP inhibitors as drugs to reduce acute kidney injury[23,24,26]. However, recent studies highlighted the beneficial role of MMPs in tissue remodeling and recovery after IRI[27,28]. Kaneko et al.[29] demonstrated that after renal IRI MMP-2 expression increased in the medulla on day 7-14 and the damaged tubules recovered within 14 days. The lack of MMP-2 expression resulted in impaired recovery of the damaged tubules, which might be due to the suppression of proliferation, migration and differentiation of regenerated tubular epithelial cells[29]. The conflicting roles of MMP expression suggest a different role for the different MMPs in the acute and recovery phase after IRI. In literature increased expression of MMPs is related to renal fibrosis[30]. However, in fasted Fibulin-$4^{+/R}$ mice MMP expression is up-regulated six months after renal IRI while there is significant less renal fibrosis compared to AL fed mice. This suggests that in the kinetics of remodeling in the injured kidney differ between Fibulin-$4^{+/R}$ and wildtype mice.

In summary, two days of preoperative fasting improved kidney function and also prevented the deterioration of kidney function and morphology, up to six months after renal IRI. Despite the 2-fold reduction in Fibulin-4, these mice seem protected against the acute injury induced by renal IRI. However, in the long-term pre-operative fasting does protect against kidney injury induced by renal IRI. Moreover, in Fibulin-$4^{+/R}$ mice, increased MMP activity after pre-operative fasting may be indicative of another protective mechanism aimed at remodeling of the kidney matrix to reduce kidney injury. These data suggest that although patients with a (familiar) history of aneurysms might not be more vulnerable to renal IRI, preoperative fasting could benefit these patients by reducing renal injury after IRI, especially in the long-term.

References

1. West CA, Noel AA, Bower TC, et al. Factors affecting outcomes of open surgical repair of pararenal aortic aneurysms: a 10-year experience. J Vasc Surg 2006;43:921-7; discussion 7-8.
2. Jongkind V, Yeung KK, Akkersdijk GJ, et al. Juxtarenal aortic aneurysm repair. J Vasc Surg 2010;52:760-7.
3. Banno H, Cochennec F, Marzelle J, Becquemin JP. Comparison of fenestrated endovascular aneurysm repair and chimney graft techniques for pararenal aortic aneurysm. J Vasc Surg 2014;60:31-9.
4. Chiesa R, Tshomba Y, Mascia D, Rinaldi E, Logaldo D, Civilini E. Open repair for juxtarenal aortic aneurysms. J Cardiovasc Surg (Torino) 2013;54:35-45.
5. Mendes BC, Oderich GS, Reis de Souza L, et al. Implications of renal artery anatomy for endovascular repair using fenestrated, branched, or parallel stent graft techniques. J Vasc Surg 2016;63:1163-9 e1.
6. Huang J, Yamashiro Y, Papke CL, et al. Angiotensin-converting enzyme-induced activation of local angiotensin signaling is required for ascending aortic aneurysms in fibulin-4-deficient mice. Sci Transl Med 2013;5:183ra58, 1-11.
7. Davis FM, Rateri DL, Daugherty A. Mechanisms of aortic aneurysm formation: translating preclinical studies into clinical therapies. Heart 2014.
8. Kaijzel EL, van Heijningen PM, Wielopolski PA, et al. Multimodality imaging reveals a gradual increase in matrix metalloproteinase activity at aneurysmal lesions in live fibulin-4 mice. Circ Cardiovasc Imaging 2010;3:567-77.
9. Moltzer E, te Riet L, Swagemakers SM, et al. Impaired vascular contractility and aortic wall degeneration in fibulin-4 deficient mice: effect of angiotensin II type 1 (AT1) receptor blockade. PLoS One 2011;6:e23411.
10. Argraves WS, Greene LM, Cooley MA, Gallagher WM. Fibulins: physiological and disease perspectives. EMBO Rep 2003;4:1127-31.
11. Hanada K, Vermeij M, Garinis GA, et al. Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. Circ Res 2007;100:738-46.
12. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest 2011;121:4210-21.
13. Snoeijs MG, van Heurn LW, Buurman WA. Biological modulation of renal ischemia-reperfusion injury. Curr Opin Organ Transplant 2010;15:190-9.
14. Kabbani LS, West CA, Viau D, et al. Survival after repair of pararenal and paravisceral abdominal aortic aneurysms. J Vasc Surg 2014;59:1488-94.
15. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010;9:40-53.
16. Verweij M, van de Ven M, Mitchell JR, et al. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation 2011;92:752-8.
17. Leemans JC, Stokman G, Claessen N, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 2005;115:2894-903.
18. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999;55:713-23.

19. Nahrendorf M, Keliher E, Marinelli B, et al. Detection of macrophages in aortic aneurysms by nanoparticle positron emission tomography-computed tomography. Arterioscler Thromb Vasc Biol 2011;31:750-7.
20. Susa D, Mitchell JR, Verweij M, et al. Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. Aging Cell 2009;8:192-200.
21. van de Ven M, Andressoo JO, Holcomb VB, et al. Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. Mech Ageing Dev 2007;128:58-63.
22. Kunugi S, Shimizu A, Kuwahara N, et al. Inhibition of matrix metalloproteinases reduces ischemia-reperfusion acute kidney injury. Lab Invest 2011;91:170-80.
23. Dejonckheere E, Vandenbroucke RE, Libert C. Matrix metalloproteinases as drug targets in ischemia/reperfusion injury. Drug Discov Today 2011;16:762-78.
24. Moser MA, Arcand S, Lin HB, et al. Protection of the Transplant Kidney from Preservation Injury by Inhibition of Matrix Metalloproteinases. PLoS One 2016;11:e0157508.
25. Basu RK, Donaworth E, Siroky B, Devarajan P, Wong HR. Loss of matrix metalloproteinase-8 is associated with worsened recovery after ischemic kidney injury. Ren Fail 2015;37:469-75.
26. Novak KB, Le HD, Christison-Lagay ER, et al. Effects of metalloproteinase inhibition in a murine model of renal ischemia-reperfusion injury. Pediatr Res 2010;67:257-62.
27. Rosell A, Lo EH. Multiphasic roles for matrix metalloproteinases after stroke. Curr Opin Pharmacol 2008;8:82-9.
28. Alwayn IP, Verbesey JE, Kim S, et al. A critical role for matrix metalloproteinases in liver regeneration. J Surg Res 2008;145:192-8.
29. Kaneko T, Shimizu A, Mii A, et al. Role of matrix metalloproteinase-2 in recovery after tubular damage in acute kidney injury in mice. Nephron Exp Nephrol 2012;122:23-35.
30. Zhao Y, Qiao X, Tan TK, et al. Matrix metalloproteinase 9-dependent Notch signaling contributes to kidney fibrosis through peritubular endothelial-mesenchymal transition. Nephrol Dial Transplant 2016.

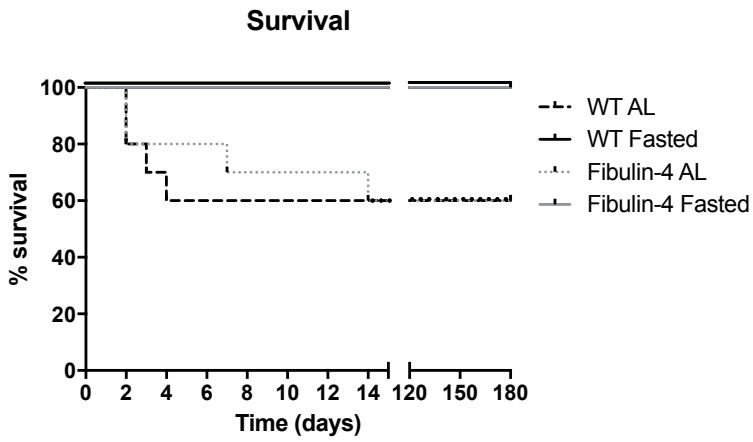


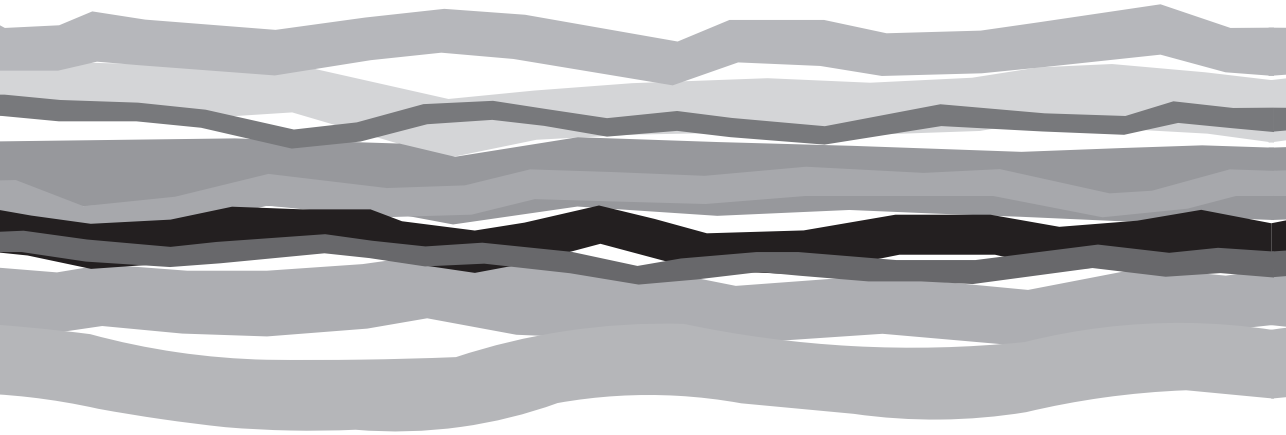
Figure S1. AL fed WT and Fibulin-$4^{+/R}$ mice had a survival of 60% within the 6 months after renal IRI, while fasted WT and Fibulin-$4^{+/R}$ mice showed a survival of 100%.

Table S1. Overview of the experimental design
(A) Overview short-term experiments

Dietary intervention	Genotype	Surgical intervention	Follow-up	Parameters measured
Ad libitum	Wild type	37min IRI	Baseline, 6 hrs, 24 hrs or 7 days	Body weight, food intake, kidney function, histology, gene expressions and survival.
Ad libitum	Fibulin-$4^{+/R}$	37min IRI	Baseline, 6 hrs, 24 hrs or 7 days	Body weight, food intake, kidney function, histology, gene expressions and survival.
2 day fasted	Wild type	37min IRI	Baseline, 6 hrs, 24 hrs or 7 days	Body weight, food intake, kidney function, histology, gene expressions and survival.
2 day fasted	Fibulin-$4^{+/R}$	37min IRI	Baseline, 6 hrs, 24 hrs or 7 days	Body weight, food intake, kidney function, histology, gene expressions and survival.

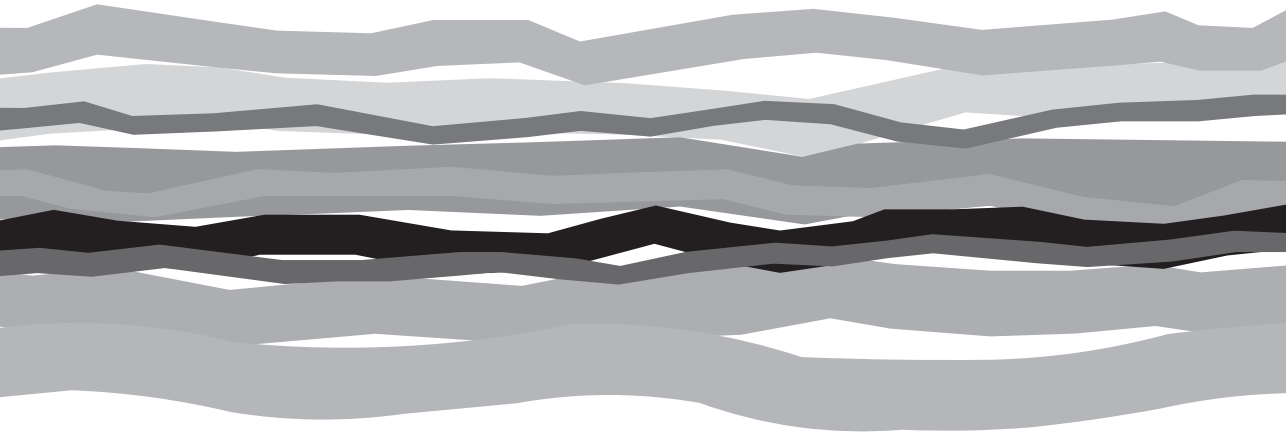
(B) Overview long-term experiments

Dietary intervention	Genotype	Surgical intervention	Follow-up	Parameters measured
Ad libitum	Wild type	30min IRI	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
Ad libitum	Fibulin-4$^{+/R}$	30min IRI	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
2 day fasted	Wild type	30min IRI	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
2 day fasted	Fibulin-4$^{+/R}$	30min IRI	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
Ad libitum	Wild type	Control	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
Ad libitum	Fibulin-4$^{+/R}$	Control	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
2 day fasted	Wild type	Control	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.
2 day fasted	Fibulin-4$^{+/R}$	Control	7 days or 6 months	Survival, body weight, food intake, kidney function (48hrs), histology, MMP expression kidney and aorta.



Chapter 9

Summary and discussion



Summary and discussion

Organ transplantation is the treatment of choice for patients with end-stage organ failure and offers the best chance on long-term survival and improvement of quality of life[1]. However, transplantation is facing a shortage of donor organs which limits its widespread use[2]. The increasing gap between availability of donor organs and demand has contributed to the expansion of the donor pool. Nowadays not only Donation after Brain Death (DBD) donors and living donors are being used, but also Donation after Circulatory Death (DCD) donors and extended criteria donors.

Another limitation of organ transplantation is the development of ischemia reperfusion injury (IRI) during transplantation. IRI is inevitable during transplantation and has major consequences for graft- and patient survival[3-6]. Although DCD kidney donation has increased the number of donor kidneys with 30% in some programs, this comes with a disadvantage[7]. DCD donors and extended criteria donors are more vulnerable to IRI since the organs suffer from prolonged warm ischemia time, increased donor age or comorbidity of the donor[8]. Therefore prevention or reduction of IRI could improve graft survival and decrease patient morbidity. Unfortunately, at this moment there is no therapy available to reduce or ameliorate IRI.

The overall aims of this thesis were to: 1) examine gene expression profiles of living-, DBD- and DCD donor organs in relation to transplant outcome, and 2) to determine if preconditioning strategies such as dietary preconditioning or the use of mesenchymal stem cells could be used as a therapeutic approach to ameliorate IRI.

In **chapter 2** we reviewed the literature on experimental and clinical studies on dietary preconditioning, preservation, ischemic pre- and post-conditioning, cell therapy, pharmacological treatment and microRNA therapy as intervention strategies to reduce renal IRI. These therapies to reduce renal IRI can be implemented in both donor and recipient, and before, during and after transplantation. Studying renal IRI is complex, coping with translational difficulties, and multifactorial pathophysiological mechanisms. Experimental research has contributed to the development of strategies to prevent or ameliorate IRI. However, despite these advances in animal studies, only few animal data are finding their way into the clinic and improve transplant outcome[9].

Due to the increasing gap between organ demand and supply, different donor types are being used. Kidneys from living donors are exposed to minimal warm and cold ischemia times, which results in a superior long-term graft survival compared to DBD- and DCD donor grafts. DBD kidneys are affected by the changes in hormonal and circulatory state, which contribute to organ damage and decreased post-transplant function and graft survival compared to living donors[10,11]. Kidneys from DCD donors suffer from prolonged warm ischemia times, which increases the incidence of delayed graft function.

Despite the higher incidence of delayed graft function in DCD kidneys compared to DBD kidneys, graft survival is similar[12]. The pathophysiology of DBD donors is relatively well established, and strategies to optimize donor organs are emerging[13]. In contrast, experimental studies on the changes that occur in DCD donor organs are sparse[14].

In **chapter 3,** expression levels of genes indicative of inflammation, cytoprotection and injury at the time of kidney retrieval, after clinically relevant cold ischemia times, and over a time-course during cold storage are compared between living donors, DBD- and DCD donors in a rat model.

The most striking finding in our study is the difference between DBD- and DCD donors in renal transcription of pro-inflammatory genes. Compared to kidneys after living donation, IL-1β, IL-6, TNF-α, MCP-1, TLR4, P-selectin and E-selectin were massively up-regulated in DBD kidneys, and only moderately increased in DCD kidneys. The high levels of pro-inflammatory and injury genes in DBD donors are in contrast with the high clinical incidence of delayed graft function in DCD- compared to DBD donors. Human DCD donors may suffer from other injuries, which influence the inflammatory response and might play an important role in the incidence of DGF and post-transplant outcome[15]. Besides that, the absence of huge inflammatory changes in our study could be an explanation why the impact of DGF is reduced in DCD kidneys.

A significant increase in the expression of injury markers Kim-1, and p21, and the cytoprotective gene HO-1 accompanied the up-regulation of pro-inflammatory genes. In living donors, up-regulation of HO-1 expression had a protective effect on renal IRI[16]. In contrast, the increased levels of HO-1 in DBD kidneys did not prevent renal damage[17]. This suggests that DBD causes renal damage to such an extent that the increased expression of HO-1 is insufficient to ameliorate the damage, and therefore may be viewed as a marker of injury[17]. After clinically relevant cold ischemia times, the gene expression profiles remained stable. However, in both post-mortem donors the average number of apoptotic cells increased between retrieval and 18 hours of cold storage.

In conclusion, we show a massive up-regulation of inflammatory, cytoprotective, and injury genes in DBD kidneys, but not in LD- or DCD kidneys at the time of graft retrieval, which remains stable during cold preservation. These results may imply why delayed graft function in DCD kidneys does not have the deleterious effect it has on DBD kidneys and lend further support to the anti-inflammatory treatment of DBD donors.

After finding the remarkable differences in expression levels of inflammatory, cytoprotective and injury genes between DBD- and DCD kidneys, we wondered if these differences were organ specific. In **chapter 4**, we compared gene expression profiles in rat livers from living donors, DBD- and DCD donors at the time of retrieval, after clinically cold ischemia times, and over a time-course during cold storage.

As mentioned before, living donors experience minimal warm and cold ischemia times[18], and DBD donors suffer from physiological perturbations that influence post-transplant outcome[11]. Organs from DCD donors are exposed to a period of hypotension, hypoxia and prolonged warm ischemia time, which affects early graft function and survival[19]. DCD Livers have an almost 11 times increased risk of developing ischemic type biliary lesions and 1.85 higher risk of graft loss, compared to DBD donors[19,20].

After retrieval, early mediators of inflammation MCP-1, HMGB1 and TLR4 were increased in DCD livers, whereas a massive up-regulation of pro-inflammatory genes IL-6, IL-1β, TNF-α, P-selectin and E-selectin was seen in DBD livers, but not in living donor livers. Cytoprotective gene HO-1 was increased in both post-mortem groups. Despite the lower expression of inflammatory genes in DCD- compared to DBD livers, the outcome after DCD liver transplantation are inferior[21]. The prolonged warm ischemia time in DCD livers might be the reason why HMGB1, marker of overall graft damage, is up-regulated directly after retrieval. Besides that, MCP-1 is considered an early mediator of inflammation and in human high levels of MCP-1 early after transplantation are associated with poor early graft function after transplantation[22] and it has been suggested that MCP-1 contributes to the development of biliary atresia[23]. After cold ischemia, DCD livers showed increased levels of MCP-1, TLR4 and HMGB1, whereas expression of pro-inflammatory genes in DBD livers remained high. During 12 hours of cold storage, mRNA gene expression profiles remain stable in LD livers, while DBD- and DCD livers show mild changes in expression of Hif-1α, Bcl-2 and HMGB1. DCD livers showed a significantly higher number of early apoptotic cells compared to DBD livers directly after retrieval. These numbers do not change during cold preservation. The higher number of apoptotic cells might explain why DCD livers have a worse outcome after transplantation, despite the lower expression of inflammatory genes. Our study shows a differential gene expression profile between livers retrieved from DBD- versus DCD donors. This suggests that the kinetics of injury and inflammatory processes might be different between DBD- and DCD donors. These differences may be relevant for more effective and early treatment in the recipient.

Long-term dietary restriction (DR) is defined as a reduction in food intake without causing malnutrition. DR is known to extend lifespan, increase health and improve stress resistance in different organisms[24]. Previously we showed that 2 and 4 weeks of 30% preoperative DR as well as three days of fasting decreased morbidity and mortality and improved kidney function in a murine renal IRI model[24]. Whether this protection is based on the reduction in calories or the source of those calories is unclear. In **chapter 5**, we investigated the role of specific macronutrients by unrestricted feeding of protein-, carbohydrate (CHO)-, and fat-free diets in inducing resistance against renal IRI.

We showed that the absence of protein for three days is sufficient to induce resistance against renal IRI, while a carbohydrate- and fat-free diet did not protect against renal IRI. In order to elucidate the mechanisms underlying these beneficial effects we produced gene expression datasets of diets proven to be either protective or not protective against renal IRI. We compared mRNA transcriptional profiles of kidneys from mice after 2 weeks of 30% DR, three days of fasting, or three days of a protein-free-, CHO-free-, or fat-free diet, before inducing renal IRI. Gene expression profiles of the non-protective CHO-free diet showed a striking overlap with gene expression profiles of the protective diets. This could either indicate that overlapping pathways are not involved in the induction of protection against renal IRI, or that they require additional changes in other pathways to induce this effect. To identify upstream regulators of these pathways, we determined the most differentially regulated transcription factors in all protective diets, which were FOXO3, HNF4A, HMGA1 and HSF1. These were not regulated in the non-protective CHO-free diet. Directly or indirectly these transcription factors are involved in stress resistance pathways[25]. Various pathways are proposed to be involved in the protective response against renal IRI generated by dietary interventions. The eukaryotic translation factor 2α (eIF2α) signaling pathway, in which eIF2α is phosphorylated by the general control nonderepressible 2 (Gcn2) kinase, might be one of them[26]. However, the role of this pathway is subject of debate[27]. In our study, up-regulation of eIF2α occurred in 3-day fasted mice only.

The nutrient sensing pathway mammalian target of Rapamycine (mTOR) mediates between growth factors, hormones and nutrients to regulate essential cellular functions. Inhibition of the mTOR pathway increases lifespan in various animal species[26]. We found a down-regulation of mTOR after 2 weeks of 30% DR and after three days of a protein-free diet. An up-regulation of mTOR was seen in the non-protective CHO-free diet and after three days of 30% DR. In contrast, MTORC1, which is a part of mTOR, was down-regulated after three days of fasting. However, more research is warranted to further elucidate the role of these transcription factors and pathways in the induction of acute stress resistance by short-term DR, which may ultimately lead to a therapeutic strategy in humans.

In mice, two weeks of 30% DR and three days of fasting does not only protect against renal-, but also against hepatic IRI. We showed that three days of a preoperative protein-free diet induces resistance against renal IRI (chapter 5). Since recent literature suggests that the beneficial effect of long-term DR on lifespan is induced by the absence of essential amino acids[28,29], in **chapter 6**, we studied the effect of essential amino acid (EAA)-free diets on hepatic IRI and aimed to find the mechanisms underlying the protective effects. Three days before inducing partial hepatic IRI, mice received a methionine (Met)-, a leucine (Leu)- or a tryptophan (Trp)-free diet. We showed that all three EAA-free

diets protected against IRI. However, a Met-free diet has a more gradual response than a Leu- and Trp-free diet, with both the Leu- and Trp-free diets showing a protective response that is activated faster after surgery than the Met-free diet. During microarray analysis these differences also come forward, since the transcriptomic response of a Met-free diet was of a lower magnitude than that of the Leu- and Trp-free diets. Since the Met-free diet resulted in the smallest number of differentially pathways and transcription factors, the analysis could be narrowed down to the results revealed by Met-free diet. Detailed analysis of overlapping genes suggested a role for pathways involved in nuclear receptor signaling, stress resistance and cell cycle regulation. The transcription factors involved are NRF2, FOXM1, SREBF2 and SMARCB1. Further in depth analysis via functional and pharmacological modulation of these factors may provide mimetics for treatment against ROS-related injury.

The liver has the unique ability of regeneration as a response to liver injury. Thanks to this remarkable feature and advances in surgical techniques, large liver resections as well as split- and living donor liver transplantation are possible. During liver transplantation and liver resection IRI is inevitable. Liver IRI leads to a decreased regenerative capacity of hepatocytes, tissue necrosis, and apoptosis[6]. IRI is a major cause of morbidity and mortality after liver transplantation and liver resection[30]. Therapies that are able to reduce hepatic IRI and promote liver regeneration could offer major benefits in both liver transplantation and resection. Mesenchymal stem cells (MSC) are able to differentiate into cell types other than their tissue of origin, secrete growth factors that stimulate other cells, and have immunomodulatory and anti-inflammatory properties[31]. Because of these characteristics, MSC are considered as a potential therapy to prevent or ameliorate hepatic IRI and stimulate liver regeneration.

In **chapter 7**, we investigated whether MSC are able to reduce hepatic IRI and stimulate liver regeneration in a combined hepatic IRI and partial hepatectomy (PH) model in the mouse. Intravenous infusion of $2x10^5$ MSC 2 hours before hepatic IRI and PH significantly decreased ASAT and ALAT levels compared to their PBS controls six hours after IRI and PH, but did not decrease the amount of hemorrhagic necrosis. In addition, MSC infused before or after hepatic IRI and PH showed no beneficial effect on liver damage and regeneration. Besides that, MSC infusion had no beneficial effect on liver regeneration, although there was a trend towards a higher liver/body weight ratio in the MSC treated animals. This is supported by a trend towards more proliferating hepatocytes and higher TFG-β expression 48 hours after IRI and PH. 3D cryo-imaging showed that intravenously infused MSC do not migrate to the damaged organ but are trapped in microcappillary networks and have a short lifespan.

In conclusion, intravenous infusion of $2x10^5$ MSC 2 hours before- or 1 hour after IRI and partial PH showed no beneficial effect on liver damage or regeneration. Many of

the infused MSC are trapped in the lungs, disappear within 2 hours and do not migrate towards the damaged liver.

Pararenal aortic aneurysms (PRAAs) are characterized by the absence of normal aorta structure between the upper extent of the aneurysm and the renal arteries, or extent to the renal arteries. The feasibility of standard endovascular aneurysm repair (EVAR) in patients with PRAAs is limited by strict anatomical requirements and high costs. Still one in five patients with PRAAs are undergoing an open repair, which leads to renal ischemia reperfusion injury (IRI), which may cause acute kidney injury. The development of aortic aneurysms is associated with extrinsic or intrinsic defects of the blood vessel wall. Aneurysms show destructive extracellular matrix remodeling, apoptosis of smooth muscle cells, and inflammatory cell infiltration.

Fibulin-4 is an elastic fiber-associated glycoprotein involved in stabilizing the organization of extracellular matrix structures and plays a role in elasticity and integrity. A reduction in Fibulin-4 expression leads to the early onset of aortic degeneration. The heterozygous Fibulin-4 mouse model (Fibulin-$4^{+/R}$) has a 2-fold reduction in Fibulin-4 expression, which results in clear aberrations in elastin formation with occasionally small aneurysm formation. In **Chapter 8** we used this mouse model to explore the effect of Fibulin-4 deficiency on renal IRI directly after surgery and in the long-term. Besides that, we studied the effect of preoperative fasting on renal IRI in Fibulin-$4^{+/R}$ and wild type (WT) control mice. Mice were fed ad libitum or fasted for 2 days before the induction of renal IRI, and animals were monitored six hours, one day or six months postoperatively.

Interestingly, before the induction of renal IRI, Fibulin-$4^{+/R}$ mice showed signs of decreased kidney function compared to WT mice. As previously described, fasted WT mice showed improved survival, kidney function and postoperative recovery compared to AL fed WT mice. In contrast, Fibulin-$4^{+/R}$ mice were protected against renal IRI compared to WT mice, and fasting could not further improve the outcome. These data might imply that mild kidney injury present at baseline in Fibulin-$4^{+/R}$ mice increased resistance against oxidative damage. In the long-term no differences were observed in survival between ad libitum (AL) fed WT and Fibulin-$4^{+/R}$ mice, suggesting that the potential survival response is only effective in reducing acute damage. Six months after renal IRI preoperative fasting improved survival in both WT and Fibulin-$4^{+/R}$ mice; all fasted mice were alive compared to only 60% of the AL fed mice. Besides that, fasting reduced kidney injury as indicated by lower serum urea levels compared to their AL fed controls. Kidneys from AL fed Fibulin-$4^{+/R}$ mice were small, dystrophic and some developed renal cysts, while none of the abnormalities were seen in fasted Fibulin-$4^{+/R}$ or WT mice. Kidney sections from fasted mice showed less tubular injury, less apoptosis and less fibrosis compared to AL fed controls. Matrix Metalloproteinases (MMPs) are responsible for extracellular matrix remodeling and directly activate cytokines, chemokines and

growth factors. Fasted Fibulin-$4^{+/R}$ mice showed increased expression levels of MMPs compared to AL fed Fibulin-$4^{+/R}$ mice. MMPs have been shown to be involved in acute and chronic renal injury and might play a role in tubular atrophy and fibrosis. However, in fasted Fibulin-$4^{+/R}$ mice MMP expression levels are up-regulated while there is significant less renal fibrosis compared to AL fed mice. This suggests a different role for MMPs in the acute and recovery phases after IRI.

In conclusion, mice predisposed to developing aortic aneurysms are protected against the short-term-, but not long-term consequences of renal IRI. Preoperative fasting protects against renal IRI and prevents deterioration of kidney function and morphology. These data suggest that preoperative fasting may decrease renal damage in patients undergoing open abdominal aneurysm repair.

Future directions

In this thesis we showed that dietary preconditioning with a preoperative 3-day protein-free or essential amino acid free diet protects against renal and hepatic ischemia reperfusion injury (IRI). These data have contributed to our understanding of a specific role for macro- and micronutrients in inducing acute stress resistance. Three days of a preoperative protein-free diet, a methionine-, leucine- or tryptophan-free diet protects against IRI due to the deficiency of the specific nutrient and not due to the reduction in calories. However, translation of these results to the clinic may be challenging due to the disparities between experimental animals and humans.

In our animal experiments, inbred, young, healthy males were used. This is obviously not representative for the patients undergoing transplantation and experience IRI. Gender, old age, overweight, comorbidities and the use of medication are variable factors that can interfere with the outcome of dietary preconditioning or other therapies that might be able to ameliorate IRI. Jongbloed et al.[32] showed that preoperative fasting also protects both male and female aged-overweight mice against renal IRI. These findings suggest a general protective response induced by dietary preconditioning against IRI regardless of age and gender. However, other studies suggest that the benefits of protein restriction are age-dependent or at least are affected by age[33,34].

Dietary preconditioning is applied before ischemia, which makes translation difficult since in the human setting, treatment before ischemia would imply treatment of the donor. This raises not only feasibility issues but also ethical concerns in DBD- and DCD donors[35]. In living donors, the donor must give full consent and treatment should not interfere with the donor's health. Ginhoven et al.[36] investigated whether a preoperative dietary restriction (DR) regimen is feasible in living kidney donors and explored the effects of DR on both the donor and recipient. Of the living kidney donors, 94% adhered

to a preoperative reduction in caloric intake. There were no differences in postoperative wellbeing, appetite and ability to perform daily tasks between the preoperative DR or control donors. Serum creatinine levels at post-operative day one and after a month showed no difference between kidneys from the DR donors or control donors. They concluded that dietary preconditioning is feasible and safe in living kidney donors, but that the dietary regimen that was used induced no major beneficial effects.

The treatment of DBD- or DCD donors is not only difficult due to the ethical concerns. Our studies suggest that the kinetics of injury and inflammatory processes might be different between the different donor types. Organs from living donors have a superior long-term outcome compared to organs from DBD- and DCD donors and express low levels of inflammatory genes. DBD donor kidneys and livers showed massive up-regulation of inflammatory and injury genes while DCD donor organs showed only mild changes. In DBD donors IL-1β, IL-6, TNF-α, MCP-1, P-selectin and E-selectin were massively up-regulated immediately after retrieval combined with the increased expression of injury markers Kim-1 and p21. DCD donors showed only a mild up-regulation of these pro-inflammatory or injury genes. These differences may be relevant for tailor made therapy as a more effective treatment of the recipient based on donor type. Recipients receiving a DBD donor organ could be treated with anti-inflammatory therapies like methylprednisolone[37] and dopamine[13] to reduce the inflammatory response. In contrast, the treatment of DCD donor organs is mainly focused on reducing IRI by using machine perfusion or normothermic regional perfusion during abdominal organ retrieval to improve the outcome after transplantation[38]. In the future, treatment of both donor and recipient should be based on donor type to improve transplant outcome.

The mechanisms underlying the beneficial effects of dietary preconditioning on IRI are still a subject of investigation while various pathways, factors and genes have been proposed to play a central role in the protective effects[25]. We analyzed the transcriptome of kidneys and livers after feeding diets proven to be either protective or not protective against IRI. Transcription factors differentially regulated in kidneys after 2 weeks of 30% DR, three days of fasting and three days of protein-free diet, but not in the non-protective carbohydrate-free diet, were FOXO3, HNF4A and HMGA1.

Phosphorylation of FOXO3 via the c-Jun N-terminal kinase (JNK)-pathway results in nuclear inclusion and activation, and regulates various processes involved in cellular stress resistance, biosynthesis, cell cycle regulation, apoptosis and autophagy[39]. The FOXO family interacts with retinoid metabolism via RXR, and is based on a co-stimulation of FOXO with PPAR. This forms a PPAR;RXR complex[40]. An indirect relation between FOXO and RXR can be established via HMGA1. The most important link between FOXO, RXR and HMGA1 is insulin signaling, because all factors are suggested to be regulators of the insulin-receptor signaling system. The beneficial effects of DR have been repeatedly

linked to the improvement of insulin sensitivity, and the link between FOXO, RXR and HMGA1 and insulin deserves further consideration[41,42].

HNF4A plays a role in the development as well as metabolism of mainly the liver and kidney[43,44]. Up-regulation of HNF4A usually depends on the presence of low levels of stress, causing an interesting phenomenon called hormesis. Hormesis is a beneficial biological response to a low dose of stressors which leads to strong adaptive changes and alter signal transduction, transcription and translation[45]. The activity of HNF4A is indirectly regulated by insulin through the action of FOXO1[45]. Activation of HNF4A results in inhibition of metabolic factors like the mammalian target of Rapamycine (mTOR)[46] and SREBF. This leads to down-regulation of the metabolism, and might improve stress resistance.

Our meta-analysis of pathways and transcriptions factors in kidneys indicates that 30% DR, 3-day fasting and three days of a protein-free diet have a significant overlap in expressed genes and pathways, which are involved in stress resistance. Unfortunately, at this moment the transcription factors are not linked to one single pathway.

After finding that three days of protein-free diet had the same robust protective effect on IRI, we investigated the role of single essential amino acid deficiency on hepatic IRI. Our results showed that all three investigated EAA-free diets protect against hepatic IRI. Our analysis showed that single EAA deprivation modifies the protective response at different levels. In line with previous results[47], all three EAA-free diets induced up-regulation of FOXO3, which is seen as one of the main contributors to the beneficial effects of dietary restriction on IRI[39].

Besides that, the EAA-free diets showed inhibition of the LXR/RXR pathway while via another pathway RXR was activated, showing an opposite effect. This might be part of a response tot the massive down-regulation of SREBF-2, as seen in all EAA-free diets, which is a main regulator of the LXR/RXR pathway[48]. When cellular sterol levels are low SREBF-2 is down-regulated, which leads to reduced LXR activity. Studies in mice showed that blocking SREBF-2 would be effective in preventing the development of hepatic steatosis and insulin resistance[49]. Our studies suggest that inhibition of SREBF-2 might play a role in the protective effect on hepatic IRI. It would be interesting to induce hepatic IRI in a SREBF-$2^{-/-}$ mouse model, to see the effect on organ function and survival.

Another effect of EAA deprivation is inhibition of the cell cycle leading to regulation of the transcription factors FOXM1. FOXM1 is associated with cell proliferation and is only expressed in proliferating cells[50]. In cancer cells, FOXM1 is highly expressed and associated with cell proliferation and protect tumor cells against oxidative stress[51,52]. Therefore, inhibition of FOXM1 is a possible agent target in tumor therapy, since inhibition of FOXM1 dysregulates the G2/M cell phase division and might make tumor cells more vulnerable for oxidative stress[51]. Contradictory to the result we show, a knockdown

for FOXM1 made human stem cells more vulnerable for oxidative stress[53]. Therefore the role of FOXM1 in protection or inducing oxidative stress is not clear and shows conflicting results.

All three EAA-free diets induced a strong activation of NRF2, both on pathway- and on transcriptional level. NRF2 is a well-known factor in DR, IRI and stress-resistance[45]. We showed that NRF2 plays an important role in the protective effect against renal IRI in young male mice after DR as well as in aged obese mice following three days of fasting[24,32,47]. NRF2 is activated by phosphorylation of eIF2α, a pathway recently implicated in the protection against IRI[45]. However, in our meta-analysis of the EAA-free diets, GCN2 and other target genes of the eIF2α pathway such as mTOR were not expressed. Phosphorylation of eIF2α via GCN2 contributes to the response seen by amino acid deprivation[54,55]. Besides that, GCN2 activation might be able to inhibit mTOR. Recently Harputlugil et al.[56] showed that the protective effect of protein restriction is not based on activation of GCN2 but activation of mTOR repressor tuberous sclerosis complex (TSC). Activation of TSC improved insulin sensitivity prior to ischemia, increased survival and reduced apoptosis after reperfusion. Future studies should determine the roles of macro- and micronutrients on the transcription factors and pathways in protection against IRI.

We also studied the use of MSC as a therapeutic approach to reduce hepatic IRI and improve liver regeneration. The effects of MSC therapy are influenced by many variables like cell number, route of administration, time of infusion and the use of different animal models. This results in different outcomes after using MSC as a therapy in IRI and liver regeneration[57-61]. How MSC exert their therapeutic effects is not clear. Do MSC migrate to the damaged organ or do MSC have an immunomodulatory or regenerative effect? We showed that MSC disappear within 2 hours after intravenous infusion, which suggests that MSC have immunomodulatory effects. MSC are able to inhibit lymphocyte proliferation and induce regulatory T and B cells[62]. Clinical trials showed that the use of MSC in humans is safe[63-65]. However, before MSC can be used as a therapy to reduce IRI and improve regeneration more research in large animal models and humans is needed. Research should be focused on the numbers of cells needed for a therapeutical effect, the route of administration and the exact time of infusion of the MSC.

As mentioned before, preoperative fasting protects against renal and hepatic IRI in a healthy mouse model. We were curious if preoperative fasting also protects against renal IRI in a Fibulin-4 deficient mouse model. Heterozygous mice (Fibulin-$4^{+/R}$) have a 2-fold reduced Fibulin-4 expression, which results in clear aberrations in elastin formation with occasionally small aneurysm formation and may therefore provide invaluable informa-

tion regarding the early onset of aortic degeneration and the effect of stressors on blood vessel walls. Before the induction of renal IRI, Fibulin-$4^{+/R}$ mice showed signs of increased kidney injury compared to WT mice. Previous studies[24,47,66] showed that preoperative fasting protects against renal IRI. However, Fibulin-$4^{+/R}$ mice were protected against short-term renal IRI and preoperative fasting could not improve this outcome. Two days of preoperative fasting improved kidney function and morphology in Fibulin-$4^{+/R}$ mice up to six months after renal IRI. These results suggest that patients with a history of aneurysms might not be more vulnerable to renal IRI and preoperative fasting could benefit these patients by reducing renal injury after IRI especially in the long-term.

In our heterozygote Fibulin-$4^{+/R}$ mouse model we studied the effect of renal IRI on kidney injury but we did not investigated if IRI induced the development of aneurysms and if preoperative fasting effects the formation of aneurysms. This could be relevant for patients with a (familiar) history of aneurysms. The expression of Fibulin-4 in human aortic walls of aortic dissection was decreased[67].

Conclusion

We found that DBD- and DCD donation induces a different inflammatory response and might need different therapeutical strategies to optimize donor quality. Besides that, we showed that a short-term protein-free or EAA-free diet protects against IRI in both kidney and liver. We attempted to unravel the underlying mechanisms of these protective diets, and found overlapping transcription factors and pathways that are possibly implicated in the protective response. More research is warranted to further elucidate the mechanisms underlying the protection of IRI by dietary preconditioning, which may ultimately lead to 'dietary restriction mimetic' therapeutic strategies that exploit the benefits of dietary restriction in humans.

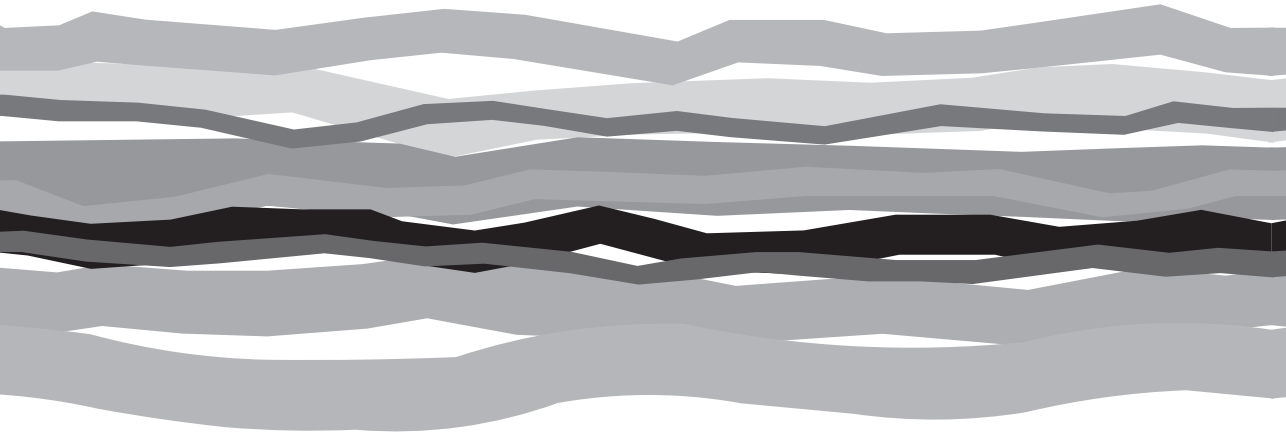
References

1. Tonelli M, Wiebe N, Knoll G, et al. Systematic review: kidney transplantation compared with dialysis in clinically relevant outcomes. Am J Transplant 2011;11:2093-109.
2. Mundt HM, Yard BA, Kramer BK, Benck U, Schnulle P. Optimized donor management and organ preservation before kidney transplantation. Transpl Int 2015.
3. Nemes B, Gaman G, Polak WG, et al. Extended criteria donors in liver transplantation Part I: reviewing the impact of determining factors. Expert Rev Gastroenterol Hepatol 2016.
4. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004;364:1814-27.
5. Kusch A, Hoff U, Bubalo G, et al. Novel signalling mechanisms and targets in renal ischaemia and reperfusion injury. Acta Physiol (Oxf) 2013;208:25-40.
6. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. World J Gastroenterol 2013;19:1683-98.
7. Yarlagadda SG, Coca SG, Formica RN, Jr., Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. Nephrol Dial Transplant 2009;24:1039-47.
8. Balaz P, Rokosny S, Wohlfahrtova M, et al. Identification of expanded-criteria donor kidney grafts at lower risk of delayed graft function. Transplantation 2013;96:633-8.
9. Sachs DH. Tolerance: of mice and men. J Clin Invest 2003;111:1819-21.
10. Westendorp WH, Leuvenink HG, Ploeg RJ. Brain death induced renal injury. Curr Opin Organ Transplant 2011;16:151-6.
11. Schuurs TA, Morariu AM, Ottens PJ, et al. Time-dependent changes in donor brain death related processes. Am J Transplant 2006;6:2903-11.
12. Nagaraja P, Roberts GW, Stephens M, et al. Influence of delayed graft function and acute rejection on outcomes after kidney transplantation from donors after cardiac death. Transplantation 2012;94:1218-23.
13. Schnuelle P, Gottmann U, Hoeger S, et al. Effects of donor pretreatment with dopamine on graft function after kidney transplantation: a randomized controlled trial. JAMA 2009;302:1067-75.
14. Laskowski IA, Pratschke J, Wilhelm MM, et al. Early and late injury to renal transplants from non-heart-beating donors. Transplantation 2002;73:1468-73.
15. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. Am J Transplant 2011;11:2279-96.
16. Nijboer WN, Schuurs TA, van der Hoeven JA, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation 2004;78:978-86.
17. Nijboer WN, Schuurs TA, van der Hoeven JA, et al. Effects of brain death on stress and inflammatory response in the human donor kidney. Transplant Proc 2005;37:367-9.
18. Kaido T, Uemoto S. Does living donation have advantages over deceased donation in liver transplantation? J Gastroenterol Hepatol 2010;25:1598-603.
19. Le Dinh H, de Roover A, Kaba A, et al. Donation after cardio-circulatory death liver transplantation. World J Gastroenterol 2012;18:4491-506.
20. Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. Ann Surg 2006;244:555-62.
21. Mathur AK, Heimbach J, Steffick DE, Sonnenday CJ, Goodrich NP, Merion RM. Donation after cardiac death liver transplantation: predictors of outcome. Am J Transplant 2010;10:2512-9.

22. Friedman BH, Wolf JH, Wang L, et al. Serum cytokine profiles associated with early allograft dysfunction in patients undergoing liver transplantation. Liver Transpl 2012;18:166-76.
23. Kobayashi H, Tamatani T, Tamura T, et al. The role of monocyte chemoattractant protein-1 in biliary atresia. J Pediatr Surg 2006;41:1967-72.
24. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010;9:40-53.
25. Longo VD, Antebi A, Bartke A, et al. Interventions to Slow Aging in Humans: Are We Ready? Aging Cell 2015;14:497-510.
26. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. Biochem J 2013;449:1-10.
27. Robertson LT, Trevino-Villarreal JH, Mejia P, et al. Protein and Calorie Restriction Contribute Additively to Protection from Renal Ischemia Reperfusion Injury Partly via Leptin Reduction in Male Mice. J Nutr 2015;145:1717-27.
28. Grandison RC, Piper MD, Partridge L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature 2009;462:1061-4.
29. Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell 2005;4:119-25.
30. Kanazawa H, Fujimoto Y, Teratani T, et al. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. PLoS One 2011;6:e19195.
31. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. Stem Cells Dev 2013;22:2825-35.
32. Jongbloed F, de Bruin RW, Pennings JL, et al. Preoperative fasting protects against renal ischemia-reperfusion injury in aged and overweight mice. PLoS One 2014;9:e100853.
33. Levine ME, Suarez JA, Brandhorst S, et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metab 2014;19:407-17.
34. Mirzaei H, Raynes R, Longo VD. The conserved role of protein restriction in aging and disease. Curr Opin Clin Nutr Metab Care 2016;19:74-9.
35. Rowinski W, Paczek L. Transplantation ethics: are we approaching the crossroads? Transplant Proc 2012;44:2171-2.
36. van Ginhoven TM, de Bruin RW, Timmermans M, Mitchell JR, Hoeijmakers JH, Ijzermans JN. Preoperative dietary restriction is feasible in live-kidney donors. Clin Transplant 2011;25:486-94.
37. Kotsch K, Ulrich F, Reutzel-Selke A, et al. Methylprednisolone therapy in deceased donors reduces inflammation in the donor liver and improves outcome after liver transplantation: a prospective randomized controlled trial. Ann Surg 2008;248:1042-50.
38. Morrissey PE, Monaco AP. Donation after circulatory death: current practices, ongoing challenges, and potential improvements. Transplantation 2014;97:258-64.
39. Wu CW, Storey KB. FoxO3a-mediated activation of stress responsive genes during early torpor in a mammalian hibernator. Mol Cell Biochem 2014;390:185-95.
40. Polvani S, Tarocchi M, Galli A. PPARgamma and Oxidative Stress: Con(beta) Catenating NRF2 and FOXO. PPAR Res 2012;2012:641087.
41. Chiefari E, Nevolo MT, Arcidiacono B, et al. HMGA1 is a novel downstream nuclear target of the insulin receptor signaling pathway. Sci Rep 2012;2:251.

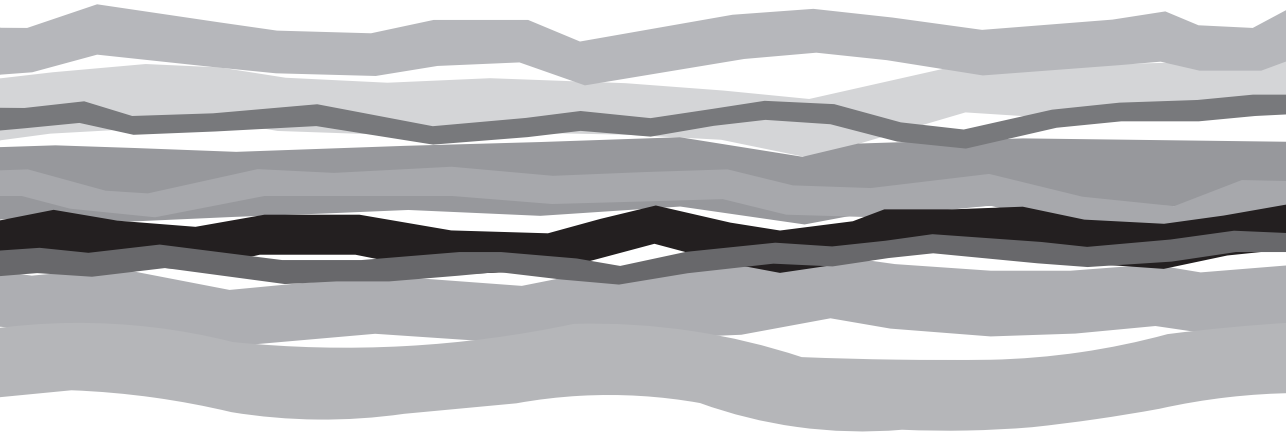
42. Mitchell SE, Delville C, Konstantopedos P, et al. The effects of graded levels of calorie restriction: II. Impact of short term calorie and protein restriction on circulating hormone levels, glucose homeostasis and oxidative stress in male C57BL/6 mice. Oncotarget 2015;6:23213-37.
43. Martovetsky G, Tee JB, Nigam SK. Hepatocyte nuclear factors 4alpha and 1alpha regulate kidney developmental expression of drug-metabolizing enzymes and drug transporters. Mol Pharmacol 2013;84:808-23.
44. Gonzalez FJ. Regulation of hepatocyte nuclear factor 4 alpha-mediated transcription. Drug Metab Pharmacokinet 2008;23:2-7.
45. Hine CM, Mitchell JR. NRF2 and the Phase II Response in Acute Stress Resistance Induced by Dietary Restriction. J Clin Exp Pathol 2012;S4.
46. Yuan M, Pino E, Wu L, Kacergis M, Soukas AA. Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. J Biol Chem 2012;287:29579-88.
47. Jongbloed F, Saat TC, Verweij M, et al. A signature of renal stress resistance induced by short-term dietary r estriction, fasting, and protein restriction. Sci Rep 2017;7:40901.
48. Bensinger SJ, Bradley MN, Joseph SB, et al. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell 2008;134:97-111.
49. Rong S, Cortes VA, Rashid S, et al. Expression of SREBP-1c Requires SREBP-2-mediated Generation of a Sterol Ligand for LXR in Livers of Mice. Elife 2017;6.
50. Raychaudhuri P, Park HJ. FoxM1: a master regulator of tumor metastasis. Cancer Res 2011;71:4329-33.
51. Halasi M, Pandit B, Wang M, Nogueira V, Hay N, Gartel AL. Combination of oxidative stress and FOXM1 inhibitors induces apoptosis in cancer cells and inhibits xenograft tumor growth. Am J Pathol 2013;183:257-65.
52. Smirnov A, Panatta E, Lena A, et al. FOXM1 regulates proliferation, senescence and oxidative stress in keratinocytes and cancer cells. Aging (Albany NY) 2016;8:1384-97.
53. Kwok CT, Leung MH, Qin J, et al. The Forkhead box transcription factor FOXM1 is required for the maintenance of cell proliferation and protection against oxidative stress in human embryonic stem cells. Stem Cell Res 2016;16:651-61.
54. Peng W, Robertson L, Gallinetti J, et al. Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. Sci Transl Med 2012;4:118ra11.
55. Lageix S, Rothenburg S, Dever TE, Hinnebusch AG. Enhanced interaction between pseudokinase and kinase domains in Gcn2 stimulates eIF2alpha phosphorylation in starved cells. PLoS Genet 2014;10:e1004326.
56. Harputlugil E, Hine C, Vargas D, Robertson L, Manning BD, Mitchell JR. The TSC complex is required for the benefits of dietary protein restriction on stress resistance in vivo. Cell Rep 2014;8:1160-70.
57. Seki T, Yokoyama Y, Nagasaki H, Kokuryo T, Nagino M. Adipose tissue-derived mesenchymal stem cell transplantation promotes hepatic regeneration after hepatic ischemia-reperfusion and subsequent hepatectomy in rats. J Surg Res 2012;178:63-70.
58. Saidi RF, Rajeshkumar B, Shariftabrizi A, et al. Human adipose-derived mesenchymal stem cells attenuate liver ischemia-reperfusion injury and promote liver regeneration. Surgery 2014;156:1225-31.
59. Behr L, Hekmati M, Lucchini A, et al. Evaluation of the effect of autologous mesenchymal stem cell injection in a large-animal model of bilateral kidney ischaemia reperfusion injury. Cell Prolif 2009;42:284-97.

60. Behr L, Hekmati M, Fromont G, et al. Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 2007;107:p65-76.
61. Wang W, Du Z, Yan J, et al. Mesenchymal stem cells promote liver regeneration and prolong survival in small-for-size liver grafts: involvement of C-Jun N-terminal kinase, cyclin D1, and NF-kappaB. PLoS One 2014;9:e112532.
62. de Witte SF, Franquesa M, Baan CC, Hoogduijn MJ. Toward Development of iMesenchymal Stem Cells for Immunomodulatory Therapy. Front Immunol 2015;6:648.
63. Perico N, Casiraghi F, Introna M, et al. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. Clin J Am Soc Nephrol 2011;6:412-22.
64. Reinders ME, Roemeling-van Rhijn M, Khairoun M, et al. Bone marrow-derived mesenchymal stromal cells from patients with end-stage renal disease are suitable for autologous therapy. Cytotherapy 2013;15:663-72.
65. Peng Y, Ke M, Xu L, et al. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. Transplantation 2013;95:161-8.
66. Verweij M, van de Ven M, Mitchell JR, et al. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation 2011;92:752-8.
67. Huawei P, Qian C, Chuan T, et al. Decreased expression of fibulin-4 in aortic wall of aortic dissection. Vascular 2014;22:35-41.



Chapter 10

Nederlandse samenvatting



Nederlandse samenvatting

Orgaantransplantatie biedt de beste kans op verbetering van kwaliteit van leven en overleving bij patiënten met eindstadium orgaanfalen. Helaas wordt het succes van deze behandeling overschaduwd door het tekort aan donororganen. Het aantal patiënten op de wachtlijst voor een donororgaan neemt toe, terwijl het aanbod van donororganen achterblijft. Het is dan ook noodzakelijk dat meer mensen in aanmerking komen als orgaandonor. Dit betekent dat er niet alleen organen van levende en hersendode donoren gebruikt worden, maar soms ook van hartdode donoren en donoren die buiten de 'normale' strikte regels vallen. Donoren die buiten de normale regels vallen zijn ouder dan 60 jaar of tussen de 50-59 jaar met 2 of meer van de volgende risicofactoren; hypertensie, serum kreatinine levels hoger dan 1.5mg/dL of overlijden ten gevolgen van een cerebrovasculaire oorzaak

Het succes van orgaantransplantatie wordt beperkt door het optreden van ischemie-reperfusie (I/R) schade tijdens de transplantatie. Tijdens ischemie is er spraken van zuurstoftekort, een gebrek aan voedingsstoffen en een opstapeling van afvalstoffen. Reperfusie is het herstellen van de bloedtoevoer waarbij zuurstofradicalen vrij komen. I/R schade heeft grote gevolgen voor de kwaliteit en levensduur van het donororgaan, en daarmee ook voor de overleving van de patiënt. Op dit moment is er geen behandeling of methode beschikbaar om I/R schade te voorkomen of te verminderen.

In dit proefschrift hebben wij onderzocht of het pre-conditioneren van het donororgaan door middel van diëten of stamcellen bescherming biedt tegen I/R schade. Daarnaast hebben wij de genexpressie profielen van organen afkomstig van de verschillende donortypen (levende, hersendode en hartdode donoren) in kaart gebracht.

In **hoofdstuk 2** geven wij een overzicht van experimentele en klinische studies waarin verschillende manieren zijn onderzocht om I/R schade van de donornier te verminderen of te voorkomen. Pre-conditionering met behulp van een dieet, preservatie van het orgaan, ischemische pre- en post-conditionering, celtherapie, medicamenteuze behandeling en het gebruik van microRNAs zijn onderzocht als mogelijke behandeling om renale I/R schade te voorkomen of te verminderen. Deze behandelingen, met als doel I/R schade van de donornier te verminderen, kunnen plaatsvinden in de donor én de ontvanger voor, tijdens of na transplantatie. Ondanks de veelbelovende resultaten in diermodellen, is het vertalen van deze resultaten naar de kliniek moeizaam. Het mechanisme onderliggend aan I/R schade is niet volledig bekend. Het ontrafelen van dit mechanisme kan nieuwe aangrijpingspunten in de behandeling van I/R schade aan het licht brengen. Daarnaast moet met het oog op klinische toepasbaarheid de interven-

ties die succesvol lijken in diermodellen vertaald worden naar de humane setting met behulp van randomized controlled trials.

Genexpressie in verschillende type donoren

De toenemende vraag naar donororganen en het achterblijvende aanbod heeft ervoor gezorgd dat er verschillende typen donoren gebruikt worden. Nieren van levende donoren worden blootgesteld aan minimale warme en koude ischemie tijden, wat resulteert in de meest optimale kwaliteit van de donornier vergeleken met hersendode en hartdode donornieren. Nieren van hersendode donoren ondergaan hemodynamische en hormonale veranderingen wat leidt tot orgaanschade, verminderde postoperatieve nierfunctie en verkorte levensduur van het transplantaat vergeleken met nieren afkomstig van levende donoren. Hartdode donornieren hebben een langere warme ischemie tijd en een hogere incidentie van delayed graft function (DGF). Ondanks de hogere incidentie van DGF vergeleken met de donornieren van hersendode donoren, is de levensduur van de nier gelijk. De pathofysiologie van hersendode donoren is goed in kaart gebracht en mogelijke behandelingen om de kwaliteit van de donororganen te verbeteren dienen zich aan. Als het om hartdode donoren gaat is er echter weinig informatie betreffende de pathofysiologie. In **hoofdstuk 3**, vergelijken we de mRNA expressie van genen representatief voor inflammatie, schade en bescherming tijdens uitname, na klinisch relevante koude ischemie tijden en gedurende koude opslag tussen levende, hersendode en hartdode donornieren in een rat model.

Opvallend in onze studie is het verschil in expressie van inflammatoire genen tussen hersendode en hartdode donoren. Expressie van de inflammatoire genen IL-1β, IL-6, TNF-α, MCP-1, TLR4, P-selectin en E-selectin was sterk verhoogd in hersendode donornieren, terwijl in hartdode donornieren deze genen in mindere mate waren verhoogd. Ondanks de sterk verhoogde expressie van inflammatoire genen in hersendode donornieren, is de incidentie van DGF hoger in hartdode donornieren terwijl de uiteindelijkel evensduur van de donornier gelijk is. Mogelijk spelen andere factoren hierbij een rol. Naast een stijging van de expressie van inflammatoire genen, vonden wij dat ook de markers voor schade, Kim-1 en p21, verhoogd waren net als het beschermende gen HO-1. In levende donoren was een verhoogde expressie van HO-1 geassocieerd met een beschermend effect op renale I/R schade. De verhoogde expressie van HO-1 in hersendode donornieren, was echter niet in staat de I/R schade te beperken. Hersendode donornieren lopen waarschijnlijk zodanig veel schade op, dat verhoogde expressie van HO-1 niet voldoende is om nierschade te reduceren. De hoogte van HO-1 lijkt wel indicatief voor de mate van schade. Na koude ischemie, blijven de gen expressie levels stabiel in alle donortypen. Desondanks, neemt in zowel hersendode als hartdode donornieren, het aantal apoptotische cellen toe ten opzichte van het aantal apoptotische cellen aanwezig bij uitname van het donororgaan.

Concluderend, laten wij zien dat in nieren die afkomstig zijn van een hersendode donor, de expressie van inflammatoire, schade-geassocieerde en beschermende genen enorm verhoogd is vergeleken met levende en hartdode donornieren. Deze gen expressie blijft stabiel tijdens en na koude preservatie. Deze resultaten suggereren dat DGF in nieren van hartdode donoren minder schadelijk is, dan DGF in hersendode donoren. In hersendode donoren kan anti-inflammatoire therapie mogelijk een behandelmethode zijn om de orgaankwaliteit te optimaliseren.

Na de opvallende verschillen tussen de expressie levels van inflammatoire, beschermende en schade markers tussen hersendode en hartdode donornieren, vroegen wij ons af of deze bevindingen orgaan specifiek zijn. Daarom hebben wij in **hoofdstuk 4,** de genexpressie profielen onderzocht in donorlevers afkomstig van levende, hersendode en hartdode ratten op het moment van uitname, na klinisch relevante koude ischemie tijden en gedurende koude preservatie. Levers van hartdode donoren hebben een 11 keer hogere kans op het ontwikkelen van ischemische galweg schade en een 1.85 keer hogere kans op orgaan falen ten opzichten van hersendode donoren.

Direct na uitname van levers afkomstig van hersendode donoren waren de inflammatoire genen IL-6, IL-1β, TNF-α, P-selectin en E-selectin sterk verhoogd, terwijl in hartdode donorlevers de vroege markers voor inflammatie, MCP-1, HMGB1 en TLR4 verhoogd waren. De donorlevers afkomstig van levende donoren lieten géén verhoging zien van de inflammatoire genen. Ondanks de lagere expressie waarden van inflammatoire genen in hartdode versus hersendode leverdonoren, was de postoperatieve uitkomst van hartdode donorlevers slechter. De langdurige warme ischemie waaraan hartdode donorlevers worden blootgesteld is mogelijk de reden waarom HMGB1, een marker voor algemene orgaanschade, verhoogd is direct na uitname van de lever. Daarnaast wordt MCP-1 gezien als een vroege inflammatoire marker en zijn verhoogde MCP-1 waarden in patiënten postoperatief geassocieerd met een slechte orgaanfunctie. Daarnaast draagt MCP-1 mogelijk bij aan de ontwikkeling van galgangatresie. Na koude ischemie bleven de vroege inflammatoire markers MCP-1, TLR4 en HMGB1 verhoogd in hartdode donorlevers terwijl in hersendode donorlevers de inflammatoire genen verhoogd bleven. Tijdens de 12 uur durende koude preservatie bleven de mRNA expressie levels stabiel in levende donorlevers terwijl in hersendode en hartdode donorlevers subtiele veranderingen in expressie van Hif-1α, Bcl-2 en HMGB1 plaatsvond. Hartdode donorlevers hadden een significant hoger aantal apoptotische cellen vergeleken met hersendode levers direct na uitname. Tijdens koude preservatie veranderde het aantal apoptotische cellen niet. Het hoge aantal apoptotische cellen in hartdode donorlevers is mogelijk een verklaring voor de slechtere postoperatieve uitkomst ondanks de lage expressie van de inflammatoire genen. Onze studie laat zien dat in levers afkomstig van hersendode en hartdode donoren de genexpressie profielen anders zijn. Dit suggereert

dat het onderliggende schade inducerende mechanisme in hersendode en hartdode donoren verschillend is. Deze uitkomsten zijn relevant voor een effectievere behandeling van de ontvanger aangepast aan donortype.

Nutritionele preconditionering

Dieet restrictie (DR) is een reductie in voedselinname zonder dat er sprake is van ondervoeding. Van langdurige DR is het bekend dat het, in verschillende soorten organismen, een toename geeft van de levensverwachting, verbetering van de gezondheid en verhoogde resistentie tegen schade veroorzaakt door zuurstofradicalen. Omdat renale I/R schade in belangrijke mate wordt veroorzaakt door zuurstofradicalen hebben wij onderzocht of DR hiertegen kan beschermen. In voorgaande onderzoeken hebben wij laten zien dat kortdurende DR voor inductie van I/R schade, namelijk 2 en 4 weken 30% DR, en 3 dagen vasten, de nierfunctie verbetert en de morbiditeit en mortaliteit in een muismodel van renale I/R schade vermindert. Of deze bescherming gebaseerd is op de reductie in het aantal calorieën of de bron van de calorieën is onduidelijk. In **hoofdstuk 5** onderzoeken wij of een eiwit-, koolhydraat- of vetvrij dieet bescherming biedt tegen renale I/R schade.

Zowel een preoperatief eiwitvrij dieet gedurende 3 dagen, 30% DR en 3 dagen vasten bieden bescherming tegen renale I/R schade, terwijl een koolhydraat- en vetvrij dieet geen bescherming bieden tegen renale I/R schade. De beschermende diëten laten een significante overlap zien tussen de mRNA transcriptie profielen en mechanismen, welke mogelijk een rol spelen in de bescherming tegen oxidatieve schade die ontstaat na renale I/R schade. Een meta-analyse van transcriptie factoren laat zien dat in de beschermende diëten de expressie van in elk geval vier transcriptie factoren verhoogd is (FOXO3, HNF4A, HMGA1 en HSF1), welke op hun beurt verschillende mechanismen kunnen activeren die een rol spelen bij de bescherming tegen oxidatieve schade. In de literatuur is een link tussen deze transcriptiefactoren en mechanismen betrokken bij stress resistentie gemaakt. Echter, meer onderzoek is nodig om een volledig beeld te krijgen van de onderliggende mechanismen die een rol spelen bij het beschermende effect van pre-conditionering middels DR, vasten of een eiwitvrij dieet met als ultiem doel het ontwikkelen van een behandeling om I/R schade te voorkomen/verminderen in het menselijk lichaam.

In muizen biedt preoperatief 2 weken 30% DR of 3 dagen vasten niet alleen bescherming tegen renale I/R schade maar ook tegen I/R schade van de lever. Wij hebben laten zien dat een preoperatief eiwitvrij dieet gedurende 3 dagen bescherming biedt tegen renale I/R schade (hoofdstuk 5). In **hoofdstuk 6** bestuderen wij het effect van een preoperatief essentieel aminozuur (EAA) vrij dieet op hepatische I/R schade en hebben wij getracht het specifieke onderliggende mechanisme van bescherming in kaart te brengen. Drie

dagen voor het induceren van hepatische I/R schade kregen muizen een methionine (Met)-, leucine (Leu)- of tryptofaan (Trp) vrij dieet. Alle drie de EAA-vrije diëten lieten minder leverschade zien na het induceren van I/R schade vergeleken met muizen die een normaal dieet kregen. Opvallend is dat een Met-vrij dieet een meer geleidelijke respons liet zien en een Leu- en Trp-vrij dieet direct een beschermend effect lieten zien. Deze verschillen zijn ook zichtbaar in de microarray analyse, waarbij de respons op het Met-vrije dieet minder sterk was ten op zichten van de respons op zowel het Leu- als het Trp-vrije dieet. Het Met-vrije dieet heeft invloed op het minste aantal transcriptie factoren, de microarray analyse kon op deze manier versmald worden. Uit deze analyse komen een aantal overlappende transcriptiefactoren naar voren zoals NRF2, FOXM1, SREBF2 en SMARCB1. Helaas hebben deze transcriptie factoren invloed op meerdere mechanismen en is er niet één overkoepelend mechanisme. Er is meer onderzoek nodig naar het exacte onderliggende mechanisme om in de toekomst een behandeling te vinden die mensen bescherming biedt tegen I/R schade.

Het effect van mesenchymale stamcellen

De lever heeft als unieke eigenschap dat het in staat is te regenereren na schade. Dankzij deze eigenschap en verbeterde chirurgische technieken, zijn leverresectie en -transplantatie middels een levende donor mogelijk. Tijdens leverresectie en –transplantatie is I/R schade niet te voorkomen. Hepatische I/R schade vermindert de regeneratieve functie van de hepatocyten en kan leiden tot necrose en apoptose. I/R schade is een risicofactor voor een verhoogde morbiditeit en mortaliteit na levertransplantatie en -resectie. Therapieën die in staat zijn I/R schade te verminderen kunnen bijdragen aan een betere postoperatieve uitkomst. Mesenchymale stamcellen (MSC) zijn in staat te differentiëren in verschillende celtypen, scheiden groeifactoren uit en hebben immunomodulatoire en anti-inflammatoire eigenschappen. Dankzij deze eigenschappen worden MSC als mogelijke behandeling gezien om I/R schade te voorkomen of te verminderen en regeneratie te stimuleren.

In **hoofdstuk 7** hebben wij in de muis onderzocht of na een combinatie van hepatische I/R schade en partiële hepatectomie (PH), MSC in staat zijn I/R schade te verminderen en regeneratie te bevorderen. Intraveneuze toediening van $2x10^5$ MSC 2 uur voor inductie van hepatische I/R schade en PH resulteerde, 6 uur postoperatief, in significant lagere ASAT en ALAT waarden vergeleken met de onbehandelde controle groep. Echter, intraveneuze toediening van MSC voor of na I/R schade en PH liet geen reductie zien van de hoeveelheid levernecrose. Ook werd er geen effect gezien van MSC op regeneratie. Door middel van 3D cryo-imaging laten wij zien dat intraveneus toegediende MSC niet naar de plek van leverschade migreren maar vastlopen in de microcapillairen van de longen, en een korte levensduur hebben. Mochten MSC een effect hebben dan is het een paracrien effect aangezien MSC niet migreren naar de plek van schade.

Intraveneuze toediening van $2x10^5$ MSC 2 uur voor of 1 uur na I/R schade en PH laat geen beschermend effect zien op I/R schade en/of lever regeneratie. Daarnaast migreren intraveneus toegediende MSC niet naar de lever maar lopen vast in de microcapillairen van de longen en verdwijnen binnen 2 uur. Om MSC in de toekomst als potentiele behandeling te gebruiken is onderzoek nodig naar de variabelen die van invloed zijn op de werking van MSC en de betrouwbaarheid hiervan.

Heterozygote Fibuline-4 muis

Aneurysmata van de aorta ontstaan onder andere door een defect in de extracellulaire matrix. Fibuline-4 is een glycoproteine dat zorgt voor stabiliteit en structuur van de extracellulaire matrix en speelt een belangrijke rol in de elasticiteit van de vaatwand. Defecten in Fibuline-4 kunnen dan ook zorgen voor aneurysmavorming. Een muis die homozygoot is voor een mutatie in het Fibuline-4 gen, heeft een Fibuline-4 expressie die vier keer lager is dan die van wild type muizen. Deze muizen laten een chaotisch netwerk zien van elastische vezels in de vaatwand, krijgen een sterk verwijde en stijve aortawand, en overlijden aan cardiovasculaire complicaties voor het bereiken van de volwassen leeftijd. Heterozygote Fibuline-4 (Fibulin-$4^{+/R}$) muizen hebben een tweemaal lagere expressie van Fibuline-4, dat zich kan uiten in het ontstaan van kleine aneurysmata op latere leeftijd. Dit muismodel biedt daardoor de mogelijkheid om het effect van oxidatieve stress te onderzoeken op de vaatwand en de gevolgen hiervan op orgaanfunctie. Bij 8-20% van de aneurysmata zijn de nierarteriën betrokken en is endovasculaire behandeling geen optie. Hierdoor ondergaat 1 op de 5 patiënten met een pararenaal aneurysma een open chirurgische behandeling. Patiënten met een verminderde Fibuline-4 expressie hebben mogelijk een verhoogd risico op een slechtere postoperatieve uitkomst wat zich kan uiten in een verminderde nierfunctie.

Renale I/R schade is onvermijdelijk bij de open behandeling van pararenale aneurysmata en is een risicofactor voor het ontwikkelen van acuut nierfalen. Voorgaande studies hebben laten zien dat preoperatief vasten een beschermend effect heeft op renale I/R schade. Vanwege deze redenen gebruiken wij in **hoofdstuk 8** het Fibuline-$4^{+/R}$ muismodel, om te zien of een reductie in Fibuline-4 expressie invloed heeft op de mate van I/R schade op korte en lange termijn. Daarnaast onderzochten wij het effect van preoperatief vasten in Fibuline-$4^{+/R}$ muizen. Vasten verbeterde de survival, nierfunctie en het postoperatieve herstel vergeleken met wild type ad libitum (AL) gevoerde muizen. Opvallend was dat AL gevoerde Fibuline-$4^{+/R}$ muizen beschermd waren tegen renale I/R schade vergeleken met wild type muizen, en dat preoperatief vasten geen additioneel verbeterend effect had op I/R schade.

Zes maanden na het induceren van renale I/R schade waren de wild type en Fibuline-$4^{+/R}$ muizen die gevast hadden allemaal nog in leven, vergeleken met 60% van de normaal gevoerde muizen. De gevaste wild type en Fibuline-$4^{+/R}$ muizen hebben

lagere serum ureum waarden vergeleken met hun AL controles. Daarnaast zijn de nieren van AL gevoede Fibuline-$4^{+/R}$ muizen kleiner, dystrofisch en een aantal van hen laat (nier) cysten zien. Geen van deze afwijkingen werd gezien in nieren van gevaste wild type of Fibuline-$4^{+/R}$ muizen. Histologie liet significant minder tubulaire schade en fibrose zien in nieren afkomstig van gevaste wild type en Fibuline-$4^{+/R}$ muizen.

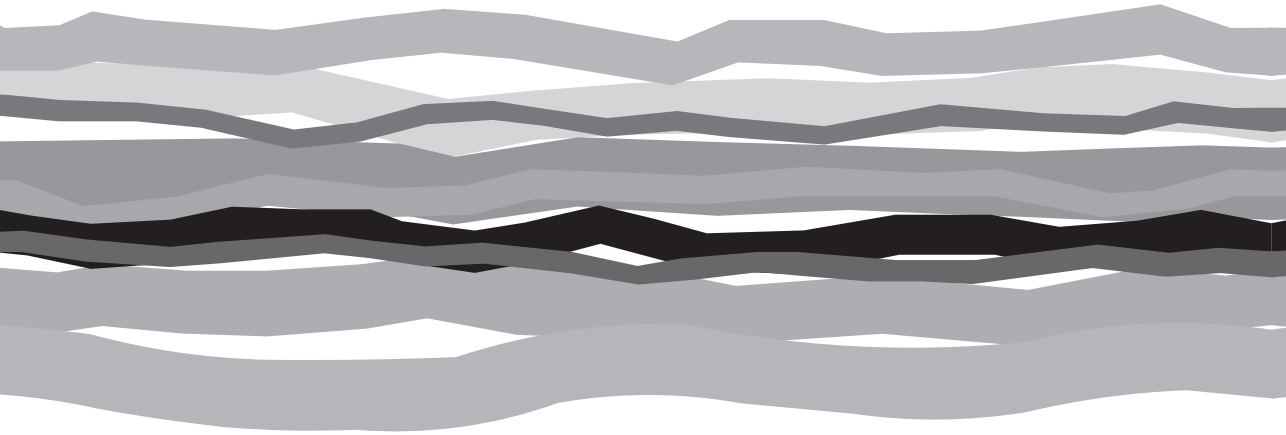
Matrix Metalloproteinases (MMPs) zijn verantwoordelijk voor het herstructureren van de extracellulaire matrix. Ze activeren onder andere cytokines, chemokines en groeifactoren. Zes maanden na het induceren van renale I/R schade was de MMP expressie in gevaste Fibuline-$4^{+/R}$ muizen verhoogd vergeleken met AL gevoerde Fibuline-$4^{+/R}$ muizen. In wild type AL gevoerde of gevaste muizen was er geen significant verschil in MMP expressie. In de literatuur is de rol van MMPs tegenstrijdig, dit suggereert dat MMPs in de acute fase na I/R schade mogelijk een andere rol spelen dan op de lange termijn. Een verhoogde MMP expressie is gerelateerd aan renale fibrose. Wij laten zien dat gevaste Fibuline-$4^{+/R}$ muizen een verhoogde MMP expressie hebben en significant minder fibrose hebben vergeleken met AL gevoerde Fibuline-$4^{+/R}$ muizen. Mogelijk speelt over-expressie van MMP in de herstelfase een rol in de regeneratie van de beschadigde nier.

Concluderend verbeterde, 2 dagen preoperatief vasten de nierfunctie op korte en lange termijn in wild type muizen. Ondanks de verminderde expressie van Fibuline-4, waren Fibuline-$4^{+/R}$ muizen beschermd tegen renale I/R schade en ontstonden er geen aneurysmata. Deze resultaten suggereren dat patiënten met een familiaire voorgeschiedenis van aneurysmata geen verhoogd risico hebben op een ernstiger beloop na renale I/R schade, terwijl preoperatief vasten de mate van I/R schade kan reduceren.

Conclusie

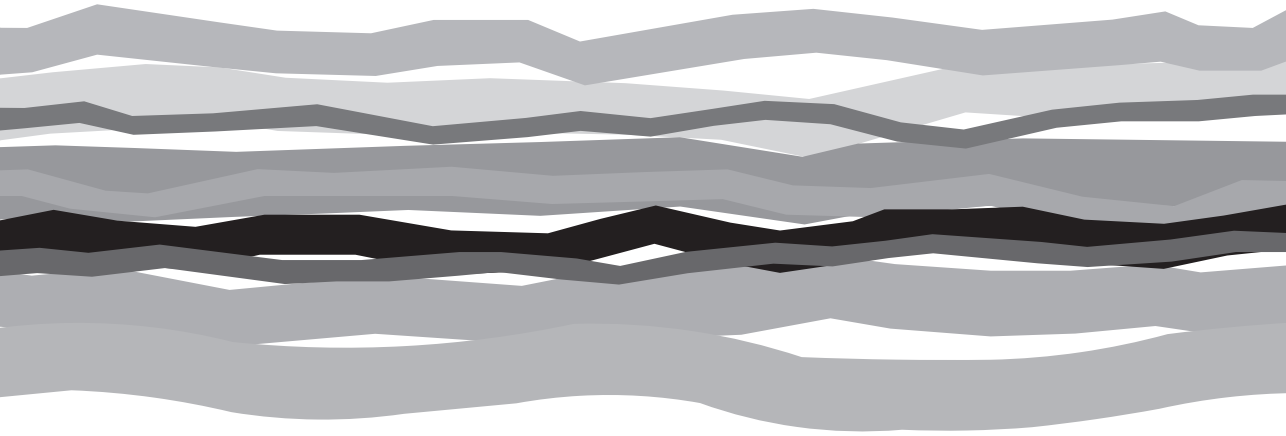
In organen van hersendode en hartdode donoren is sprake van een verschillend inflammatoir proces. Mogelijk zijn hierdoor de therapeutische behandelingen om de donor kwaliteit te optimaliseren verschillend.

Daarnaast hebben wij aangetoond dat een preoperatief eiwitvrij dieet gedurende 3 dagen bescherming biedt tegen zowel renale als hepatische I/R schade. Doormiddel van microarray analyses hebben wij getracht het onderliggende mechanismen te ontrafelen. De verschillende beschermende diëten laten een overlap zien in een aantal transcriptiefactoren en meerdere mechanismen die mogelijk betrokken zijn bij het beschermende effect. Meer onderzoek is nodig om het onderliggende mechanisme in kaart te brengen, wat uiteindelijk kan leiden tot een behandeling in de kliniek.



Appendices

I. Dankwoord
II. List of publications
III. Curriculum Vitae
IV. PhD portfolio



Dankwoord

Het heeft 'even' geduurd maar het is eindelijk zo ver, mijn proefschrift is af! Dit proefschrift is tot stand gekomen door de steun, betrokkenheid en inzet van velen. Een aantal mensen wil ik in het bijzonder bedanken.

Mijn promotor, beste prof. dr. J.N.M. IJzermans, na het doen van mijn keuzeonderzoek als onderdeel van de studie geneeskunde kreeg ik de kans om als promovenda aan de slag te gaan bij de afdeling Heelkunde. Dankzij de vrijheid en het vertrouwen dat ik kreeg kon ik de eerste twee jaar van mijn promotieonderzoek combineren met wedstrijdroeien, het beste van twee werelden, waarvoor veel dank!

Mijn copromotor, dr. R.W.F. de Bruin, beste Ron, hartelijk dank voor jouw begeleiding tijdens de eindeloze experimenten, de bemoedigende woorden tijdens tegenslagen en je oneindige geduld. Dat laatste is zeker niet mijn beste eigenschap en tijdens mijn promotietraject flink op de proef gesteld. Desondanks had jij er altijd vertrouwen in dat het 'ooit' goed zou komen, gelukkig had je gelijk en is het dan eindelijk zo ver!

Dr. M. Hoogduijn, beste Martin, het kweken van mesenchymale stamcellen bleek een uitdaging. Gelukkig was jij, samen met jouw team, bereid om te helpen. Hartelijk dank voor de prettige samenwerking, je hulp bij het schrijven van het artikel en je deelname in de commissie.

Dr. J. Essers, beste Jeroen, hartelijk dank voor de samenwerking tussen onze beide laboratoria. Jouw kritische vragen zijn van groot belang geweest bij het uitvoeren van de experimenten en het schrijven van het artikel. Bedankt voor je deelname in de commissie.

Dr. M.E.T. Dolle, beste Martijn, de microarray analyses en bijbehorende fenotypische data zijn de meest bediscussieerde onderdelen van mijn proefschrift. Inmiddels is er een mooie publicatie uit voortgekomen. Heel erg bedankt voor alle hulp en je deelname in de commissie. Ik wil ook alle betrokkenen van het RIVM bedanken voor alle hulp.

Ik wil prof. dr. H.J. Metselaar, prof. dr. C.C. Baan en prof. dr. H.G.D. Leuvenink hartelijk danken voor het beoordelen van mijn proefschrift en voor hen deelname in de oppositie.

Dr. I. van der Pluijm, beste Ingrid, het leek zo simpel om preoperatief vasten en renale ischemie-reperfusie schade toe te passen in heterozygote Fibuline-4 muizen. Toch bleek

dit nog veel voeten in aarde te hebben. Bedankt voor het laagdrempelige overleg, je kritische blik, hulp bij het analyseren van de vele data en het schrijven van het manuscript.

Alle overige co-auteurs van de publicaties in dit proefschrift: veel dank voor jullie bijdrage en kritische blik.

Ik wil alle collega's van het laboratorium voor Experimentele Chirurgie bedanken voor de gezellige tijd, hulp bij het brainstormen en uitvoeren van de experimenten. Daarnaast wil ik alle collega's bedanken die de afgelopen jaren op kamer Ee-173 hebben gezeten!

Mariëlle, bedankt voor de begeleiding tijdens mijn keuzeonderzoek, dit is de basis geweest voor mijn promotie! Met name ben ik dankbaar voor de tip om alle data netjes te ordenen, hierdoor kan ik zelfs van de eerste experimenten alle data terugvinden!

Sandra, vanaf het eerste moment heb jij mij geholpen met het uitvoeren van alle experimenten. Mijn ambitieuze, strakke planningen zag jij als een uitdaging. Samen lukte het ons altijd alles binnen de tijd af te krijgen! Heel veel dank voor al je hulp en gezelligheid!

Gisela, jij kwam bij ons op het lab werken toen ik eigenlijk al 'klaar was', echter moest er zo nu en dan toch nog iets aanvullends gedaan worden en het liefst snel. Bedankt voor je hulp in de laatste fase van mijn promotietraject.

Sander en Eline, vanaf het begin mijn partners in crime op 'Labje #1'. Ik wil jullie vooral bedanken voor het lachen! Sander, jouw zomerse hitjes en relaxte humeur waren een (welkome) afleiding. Eline, ons gezamenlijke doel om Sander met regelmaat voor de gek te houden zorgde altijd voor een boel voorpret! Veel succes met jullie carrière.

Franny, eindelijk is het af! De twee stukken die wij samen hebben geschreven zijn denk ik de meest besproken (hoofdpijn) stukken van mijn boekje. Het leek soms of er nooit een eind aan zou komen; hier wat toevoegen, daar weer wat af, nieuwe analyses, ga zo maar door. Mede dankzij jouw doorzettingsvermogen is het toch gelukt! Heel veel succes met het afronden van je promotie onderzoek!

Maren, mijn allerliefste roomie, zonder jou als paranimf zou mijn promotie niet compleet zijn. Als huisgenoten begonnen en als soulmates geëindigd! De vele koppen koffie, alcoholische versnaperingen, urenlange gesprekken en bovenal het lachen samen (helaas snapt niet iedereen onze humor) zijn mij zeer dierbaar. Ik hoop op nog vele jaren lachen samen. Er bestaat geen twijfel dat jij jouw promotieonderzoek bij de MDL tot een goed einde gaat brengen en een fantastische MDL-arts zal worden!

Christa, lief vriendinnetje, wie had ooit gedacht dat je van 'korfbal vriendinnetje' naar paranimf zou promoveren?! Ondanks dat je geen idee had wat een paranimf was, zei je zonder enige twijfel JA! Ruim 20 jaar kennen wij elkaar, partners in crime op de middelbare school, tijdens schoolfeesten en later in Rotterdam! Jouw optimisme, relativeringsvermogen, kracht en humor zijn heerlijk! Met jou wil ik nog lang 'viben' en Berlijn onveilig maken!

Lieve meiden, mijn tweede familie, het is eindelijk zover: we kunnen aan de champagne! Sinds de middelbare school zijn jullie mijn trouwste vriendinnen. Lief en leed delen wij inmiddels ruim 15 jaar met elkaar. De afgelopen jaren zijn mijn frustraties omtrent onderzoek met regelmaat besproken tijdens de borrel. Ik ben ontzettend trots dat ik het eindresultaat met jullie mag delen en dat jullie een keer op mijn kosten kunnen borrelen! Op naar meer herinneringen, avonturen en dansjes!

Lief broertje, lieve Rein, 'waarom doe je iets wat je niet leuk vindt', waren jouw woorden tijdens mijn klaagzang over onderzoek. Jouw nuchtere kijk op dingen is voor mij als control freak soms lastig te volgen. Jouw (Rotterdamse) uitspraken, belevenissen en humor zijn bij ons thuis legendarisch en zorgen met regelmaat voor buikpijn van het lachen. Ik ben trots op jou als grote, kleine broer!

Lieve paps en mams, één ding is zeker, zonder jullie onvoorwaardelijke liefde en steun zou dit boekje er niet zijn. Ik weet niet wie er meer opgelucht is dat het af is, jullie of ik. Niet alleen tijdens mijn studie en onderzoek maar ook langs de roeibaan waren jullie mijn trouwste supporters. Paps, onze fietstripjes blijven voor mij het hoogtepunt van het jaar, hoe gaaf dat wij dit samen delen! Gezellige gesprekken, scheldend een berg op fietsen (ik dan) en heel veel lachen! Ik kijk uit naar onze volgende uitdaging!

Mams, jij ben oprecht de allerliefste moeder ooit, niks is te gek voor jou. Bevriezen in Boston omdat ik in de sneeuw moet roeien, de auto overvol laden met Ikea spullen of last minute mee willen eten, alles kan! Lieve ouders, dankzij jullie liefdevolle opvoeding, arbeidsethos en no-nonsens instelling ben ik zo ver gekomen! Bedankt voor al jullie hulp en steun de afgelopen tijd! Ik ben zo ontzettend trots op jullie!

Allerliefste Hugo, eindelijk is het veel besproken boekje af! Ik wil je heel erg bedanken voor je eeuwige geduld, relativeringsvermogen en bovenal je steun. Letterlijk en figuurlijk hou jij mij uit de wind. Met jou deel ik mijn passie voor sport kijken en zelf sporten, voor ons is de Tour de France kijken een full time job. Ik ben benieuwd welke sportieve uitdaging je nu weer aan gaat, jij kan alles! Het leven met jou samen is zoveel leuker!

List of publications

T.C. Saat, E.K. van den Akker, J.N.M. IJzermans, F.J.M.F. Dor, R.W.F. de Bruin.
Improving the outcome of kidney transplantation by ameliorating renal ischemia reperfusion injury: Lost in translation? *Journal of Translational Medicine 2016; 14(1):20*

T.C. Saat, D. Susa, H.P. Roest, N.F.M. Kok, S. van den Engel, J.N.M. IJzermans, R.W.F. de Bruin.
A comparison of inflammatory, cytoprotective and injury gene expression profiles in kidneys from brain death and cardiac death donors. *Transplantation 2014; 15;98(1):15-21*

T.C. Saat, D. Susa, N.F.M. Kok, S. van den Engel, H.P. Roest, L.J.W. van der Laan, J.N.M. IJzermans, R.W.F. de Bruin. Inflammatory genes in rat livers from cardiac- and brain death donors. *Journal of Surgical Research 2015; 198(1):217-27*

T.C. Saat, F. Jongbloed, M. Verweij, C. Payan-Gomez, J.H.J. Hoeijmakers, S. van den Engel, C.T. van Oostrom, G. Ambagtsheer, S. Imholz, J.L.A. Pennings, H. van Steeg, J.N.M. IJzermans, M.E.T. Dollé, R.W.F. de Bruin. A signature of renal stress resistance induced by short-term dietary restriction, fasting, and protein restriction. *Scientific Reports 2017; Jan 19;7:40901*

T.C. Saat, S. van den Engel, W. Bijman-Lachger, S.S. Korevaar, M.J. Hoogduijn, J.N.M. IJzermans, R.W.F. de Bruin. Fate and effect of intravenously infused mesenchymal stem cells in a mouse model of hepatic ischemia reperfusion injury and resection. *Stem Cells International 2016; 2016:5761487*

Curriculum Vitae

Tanja Charlotte Saat werd geboren op 1 juli 1988 te Rotterdam, Nederland. In 2006 slaagde zij voor haar VWO-eindexamen aan het Emmauscollege te Rotterdam. Via de Decentrale Selectie werd zij toegelaten tot de studie geneeskunde aan de Erasmus Universiteit in Rotterdam. Na het behalen van haar Doctoraal in 2010, begon zij als onderzoeker aan haar promotieonderzoek bij de afdeling experimentele chirurgie aan de Erasmus Universiteit te Rotterdam onder leiding van prof. J.N.M. IJzermans en dr. R.W.F. de Bruin. Tijdens haar studie werd zij lid bij de Algemene Rotterdamse Studenten Roeivereniging Skadi waar haar passie voor roeien ontstond. Zij ging wedstrijdroeien en heeft dit fanatiek gedaan tijdens haar studie en eerste jaren van haar promotieonderzoek. De liefde voor sport kijken en zelf sporten is blijven bestaan.

In augustus 2014, na drie maanden reizen door China, Tibet en Nepal, ging zij verder met de studie geneeskunde en begon zij aan haar coschappen. Haar oudste coschap heeft zij bij de chirurgie in het Maasstad gevolgd, haar keuze-coschap op de Maag-, Darm-, Leverafdeling in het Erasmus MC. Na het behalen van haar artsexamen in 2016 is zij begonnen als ANIOS bij de Interne geneeskunde in het Franciscus Gasthuis. Zij wil uiteindelijk graag Maag-, Darm-, Leverarts worden.

PhD Portfolio

Name PhD student: Tanja Charlotte Saat
PhD period: November 2010 – December 2013
Erasmus MC Department: Surgery **Promotor:** Prof. dr. J.N.M. IJzermans
Research School: Molecular Medicine **Co-promotor:** Dr. R.W.F. de Bruin

1. PhD training		
	Year	**Workload (ECTS)**
General courses		
- Laboratory animal science	2011	5.7
- Biomedical English Writing (short)	2011	2.0
- Basic introduction course on SPSS	2011	1.0
Seminars and workshops		
- Photoshop and Illustrator CS4 workshop	2011	0.3
- Presenting Skills for Scientists	2011	1.0
Presentations		
- National conferences	2010	1.0
	2011	4.0
	2012	2.0
	2013	2.0
	2014	1.0
- International conferences	2011	1.0
	2013	7.0

2. Teaching activities		
- Supervising Bachelor thesis (Higher Laboratory education)	2013	5.0
- Supervising Bachelor thesis (Higher Laboratory education)	2013	5.0
- Supervising Bachelor thesis (Higher Laboratory education)	2014	5.0