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Studying the short-term complications of kidney transplantation: from bed to bench

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Studying the short-term complications of kidney transplantation: from bed to bench

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de Klim

*Het Nationaal Donor Monument 'de Klim' staat symbool voor een getransplanteerde die dankzij zijn donor naar een nieuw leven klimt en een nabestaande die uit een dal van verdriet klimt omdat de overleden donor in iemand anders voortleeft. **

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Chapter 1

General introduction, objectives and outline of this thesis

Michèle J.C. de Kok

General introduction

In patients with end-stage renal disease, renal replacement therapy is a prerequisite for survival and can be achieved by hemodialysis, peritoneal dialysis or kidney transplantation. Although the preferred option of renal replacement modality should be based on patient-centered medical practice, kidney transplantation is the most preferred option due to its advantages in terms of patient survival, quality of life and healthcare costs.[1-3]

One of the first successful kidney transplantation in humans was performed in 1954 between twin brothers in Boston, USA,[4] and ever since, organs retrieved from living donors and donation after brain death (DBD) donors have provided the vast majority of kidney grafts for transplantation globally.[5] However, its medical success as well as the increasing patient population (due to an ageing population and an increasing prevalence of kidney failure) has led to a substantial mismatch between kidney transplant demand and supply.[6,7] This is reflected by long waiting lists and has resulted in the death of many patients while waiting for kidney transplantation.

In an effort to reduce the waiting lists for kidney transplantation, transplantation of kidneys retrieved from controlled donation after circulatory death (DCD) donors has been proposed as an effective strategy to expand the donor pool.[5,8] In DCD donors, retrieval of organs for transplantation purposes is followed after death confirmed using circulatory criteria, and differs from DBD donors in whom death has been declared using neurological criteria.[9] As a consequence, DCD donors are, as opposed to DBD donors, exposed to an inevitable period of warm ischemia prior to laparotomy and cold flush (Figure 1).

Despite long-standing arguments that DCD kidney grafts may reduce the donor organ shortage, many countries remain highly reluctant toward the use of DCD grafts.[7,10] While for some countries this is based on legal restrictions, ethical issues or logistical concerns,[7] for the majority of countries this reticent attitude toward the use of DCD grafts generally reflects medical concerns that are based on a high incidence of early graft loss (EGL) and delayed graft function (DGF).[10-12] Whilst the loss of a kidney graft shortly after transplantation (i.e. EGL) is an obvious disastrous complication, there is also concern about DGF (the delayed functional recovery of a kidney graft as a clinical manifestation of ischemia-reperfusion (I/R) injury) as it has been associated with impaired renal function and impaired long-term graft survival.[13,14]

In the first part of this thesis, I will focus on whether the perception in clinical practice that transplantation of DCD kidneys is inferior to DBD kidneys and associated with more posttransplant complications is still correct. Obviously, if incorrect, the medical

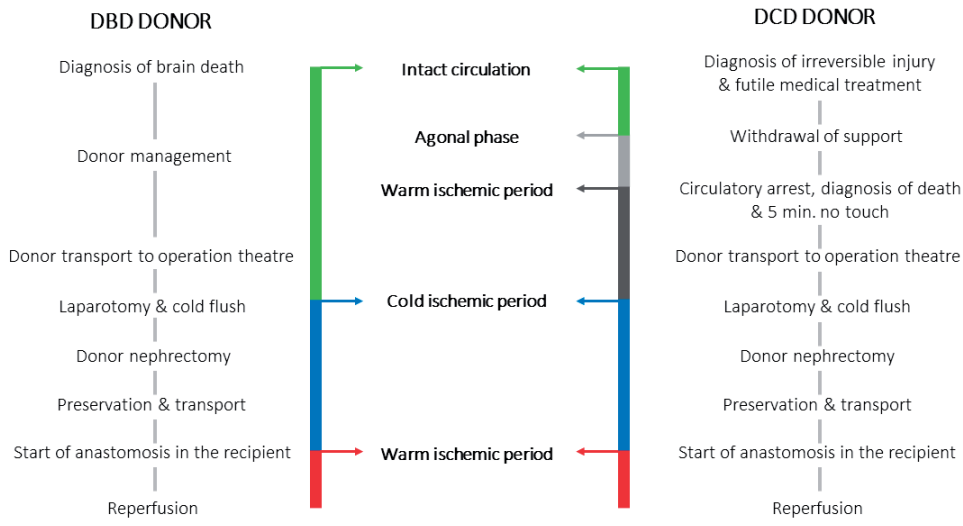

Figure 1. Clinical pathways of kidney organ donation after brain death (DBD) and circulatory death (DCD).

argument to abstain and not attempt to increase utilization of DCD kidneys is not justified. In the second part of this thesis, I will move from bed to bench in order to further elaborate on DGF (defined as the situation in which the recipient is temporarily dialysis dependent after transplant surgery) as the primary complication in DCD kidney transplantation and explore the challenges, opportunities and future perspectives in research focusing on I/R injury.

Objectives and outline of this thesis

Part I

While high incidences of EGL and DGF are considered major impediments to a more liberal use of DCD grafts, data from The Netherlands and the United Kingdom (in which DCD grafts currently account for ±50% of all deceased-donor kidney transplant procedures) show similar long-term survival outcomes after DBD and DCD kidney transplantation (Figure 2).[10,15,16] These observations are remarkable and contradict with the general perception that transplantation outcomes of DCD grafts are inferior to DBD grafts. Moreover, an explanation for contrasting short-term outcomes but equivalent long-term outcomes is lacking. In **the first part of this thesis**, we therefore aim to explore potential explanations for equivalent long-term survival outcomes of DBD and DCD kidney transplantation using an epidemiological approach.

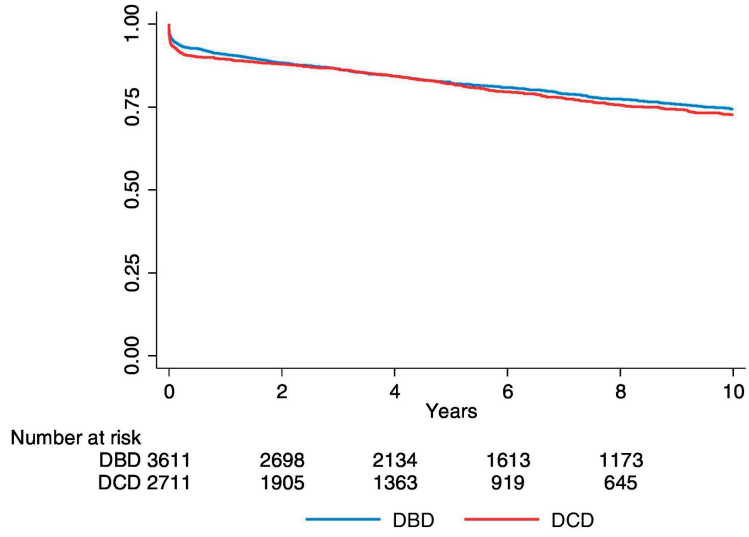

Figure 2. Recipient death censored 10-year graft survival of donation after brain death (DBD) and circulatory death (DCD) kidney grafts transplanted between January 1st 2000 and January 1st 2017 in The Netherlands. This figure was adapted from Schaapherder et al. [16]

First of all, following general introduction in **chapter 1** on deceased donor kidney transplantation, we focus in **chapter 2** on EGL as one of the most feared complications after DBD, but in particular in DCD kidney transplantation. Whilst in an era of severe organ shortages, many donor kidneys with an anticipated risk of EGL are declined for transplantation, the actual clinical consequences of EGL are currently unknown. Thus, in an attempt to estimate the optimal trade-off where the impact of EGL is balanced by the donor pool size, we first will perform a systematic analysis of the clinical consequences of early graft loss after deceased donor kidney transplantation in **chapter 2**. In **chapter 3** and **chapter 4** we subsequently aim to find an explanation for the contrasting short-term, but equivalent long-term survival outcomes of DBD and DCD kidney transplantation. In **chapter 3** we hypothesize that concerns regarding inferior DCD outcomes, often based on data from historical cohorts, are interfered by time-related effects and may therefore not apply anymore in our current era. To explore a possible effect of time on outcomes, we perform a time-dependent comparative analysis, and examine the current results achieved when transplanting kidneys from DBD and DCD donors. Next, in **chapter 4** we will search an explanation for the remarkable finding of chapter 3 that recipients of DBD and DCD grafts show graft survival equivalence, despite a persistent higher incidence of DGF in DCD grafts in the current timeframe. In this chapter we investigate whether this apparent paradox can be explained by differential impacts of DGF on DBD and DCD graft survival, and in addition we will attempt to explore its biological basis.

Part II

In the second part of this thesis, we further concentrate on DGF as in many countries–even in an era of severe organ shortages–DGF remains a main reason to not or only cautiously allow DCD programs. Although the impact of DGF on graft survival may be less than commonly thought in DCD grafts (part I), DGF is still feared as it may negatively impact the short-term outcomes (i.e. prolonged hospitalization, increased healthcare costs, and impaired graft function).[13,14] Also, it should be stressed that DGF remains–albeit less prevalent–an important complication after DBD kidney transplantation with a delayed functional recovery that is associated with a lower graft survival rate.[16,17] Thus, in an effort to reduce the incidence of DGF, we move in the **second part of this thesis** to a more molecular approach, composing a theoretical model of the pathophysiology underlying DGF as a manifestation of I/R injury, and subsequently explore emerging challenges that will arise in *in vivo* and *in vitro* studies of I/R injury.

Although the pathophysiology of I/R injury is complex and not yet fully understood, recent reports indicate that I/R injury relates to critical metabolic deficiencies due to mitochondrial damage.[18-20] It is interesting, that although several studies have identified promising interventions ameliorating the detrimental effects of I/R injury, the majority of therapies is restricted to the preclinical setting, and a definitive solution treating or even preventing I/R injury is still missing in clinical practice.[21,22] In **chapter 5** we present a novel theoretical model and provide new insights into the pathophysiology of I/R injury in a clinical setting focusing on reductive stress. However, to further dissect this theory, more research is needed. While several avenues of research (e.g. clinical, preclinical and cell culture studies) are considered suitable to study the processes of I/R injury, each type comes with its own challenges and will be discussed in **chapters 6 and 7**. Although clinical studies are generally considered most desirable for future I/R studies, the availability of human tissue for research purposes remains limited and governance surrounding operational, legal or ethical access to obtain tissue are challenging. An alternative approach to unravel underlying processes of I/R injury is the use of preclinical (i.e. animal) studies. In the context of the existing translational gap,[21,22] **chapter 6** will elaborate on the question to what extent preclinical models of renal I/R injury can mimic the clinical setting, with a particular focus on methodological challenges and interspecies differences in metabolism. Given the putative interspecies metabolic differences as well as the limited availability of human samples for research purposes, we will also focus on cell culture experiments as another promising technique to study the pathophysiology of I/R injury.

Chapter 7 highlights that metabolic (I/R) studies under cell culture conditions are profoundly interfered by the Crabtree effect, a phenomenon that describes the non-physiologic metabolic switch of cultured cells to a glycolytic phenotype.[23] Hence,

metabolic studies in cultured cells critically rely on reversal of this Crabtree effect. In **chapter 7** we therefore provide a systematic review of reported strategies aiming to circumvent the Crabtree effect in cell cultures. We also provide a critical appraisal of these strategies and present some recommendations for future research focusing on I/R injury and cellular metabolism.

Part III

In the third and final part of this thesis the summary, future perspectives and conclusions of this thesis are presented (**chapter 8**).

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PART I

The clinical impact of early graft loss and ischemia-reperfusion injury

Chapter 2

A nationwide evaluation of deceased donor kidney transplantation indicates detrimental consequences of early graft loss

Michèle J.C. de Kok, Alexander F.M. Schaapherder, Jacobus W. Mensink, Aiko P.J. de Vries, Marlies E. Reinders, Cynthia Konijn, Frederike J. Bemelman, Jacqueline van de Wetering, Arjan D. van Zuilen, Maarten H.L. Christiaans, Marije C. Baas, Azam S. Nurmohamed, Stefan P. Berger, Rutger J. Ploeg, Ian P.J. Alwayn, Jan H.N. Lindeman

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Abstract

Early graft loss (EGL) is a feared outcome of kidney transplantation. Consequently, kidneys with an anticipated risk of EGL are declined for transplantation. In the most favorable scenario, with optimal use of available donor kidneys, the donor pool size is balanced by the risk of EGL, with a trade-off dictated by the consequences of EGL. To gauge the consequence of EGL we systematically evaluated its impact in an observational study that included all 10,307 deceased-donor kidney transplantations performed in The Netherlands between 1990 and 2018. Incidence of EGL, defined as graft loss within 90 days, in primary transplantation was 8.2% (699/8,511). The main causes were graft rejection (30%), primary non-function (25%), and thrombosis or infarction (20%). EGL profoundly impacted short- and long-term patient survival (adjusted hazard ratio; 95% confidence interval: 8.2; 5.1-13.2 and 1.7; 1.3-2.1, respectively). Of the EGL recipients who survived 90 days after transplantation (617/699) only 440 of the 617 were relisted for re-transplantation. Of those relisted, only 298 were ultimately re-transplanted leading to an actual re-transplantation rate of 43%. Noticeably, re-transplantation was associated with a doubled incidence of EGL, but similar long-term graft survival (adjusted hazard ratio 1.1; 0.6-1.8). Thus, EGL after kidney transplantation is a medical catastrophe with high mortality rates, low relisting rates, and increased risk of recurrent EGL following re-transplantation. This implies that detrimental outcomes also involve convergence of risk factors in recipients with EGL. The 8.2% incidence of EGL minimally impacted population mortality, indicating this incidence is acceptable.

Introduction

Early graft loss (EGL), including primary non-function is considered a catastrophic outcome of kidney transplantation. As a consequence, when donor kidneys are expected to have an increased risk of EGL, they are declined for transplantation. Although a permissive policy toward anticipated high-risk organs will result in an unacceptable high incidence of EGL, a more reticent attitude will compromise the donor use and, as such, contribute to increasing organ shortages and longer waiting list times. Consequently, in a scenario with optimal utilization of available donor kidneys, the size of the donor pool is balanced by the risk of EGL, with the trade-off dictated by the impact of EGL.
To date, only two single-center studies have evaluated the consequences of EGL after kidney transplantation.[1,2] The authors concluded that EGL had a detrimental impact on short- and long-term patient survival. However, the low number of EGL cases did not allow an in-depth evaluation.[1,2]
Given the persistent donor organ shortage and the need to expand the donor pool without compromising outcomes, we considered a systematic, adequately powered evaluation of the clinical impact of EGL to be of importance. Therefore, we performed an in-depth, nationwide systematic analysis of the consequences of EGL in a cohort of 10,307 deceased donor kidney transplant procedures.

Materials and methods

Study population

This study was approved by the local ethics committee of the Leiden University Medical Center. Data from all 11,415 deceased donor kidney transplant procedures performed between 1990 and 2018 were retrieved from the Netherlands Organ Transplant Registry (NOTR). This nationwide, mandatory registry contains the data of all eight Dutch kidney transplant centers. Registry follow-up is conducted at three months and one year after transplantation and annually thereafter. Procedures in recipients younger than 12 years of age (n = 261), combined organ procedures (n = 635) and uncontrolled circulatory death donor procedures (Maastricht category I: dead on arrival and category II: unsuccessful resuscitation) (n = 212) were excluded. The remaining 10,307 deceased donor kidney transplants were included in the analyses. For validation purposes and for correction of missing data, additional data of recipients with EGL was retrieved from Eurotransplant and Renal Replacement Registry (RENINE), the mandatory dialysis registry of The Netherlands, and incorporated in the final database.

Eurotransplant data for the 2009-2018 interval indicate a 1-year waiting list mortality of 11.03 ± 1.41 % per year (mean ± standard deviation (SD)) for patients on the active

waiting list (kidney only) in The Netherlands,[3] implying a relative risk of death of 2.51 compared with those successfully transplanted (observed 1-year mortality rate, 4.40%). Based on this relative risk, waiting list survival curves were constructed using the Kaplan-Meier method to estimate waiting list survival times and 10- to 90-percentile intervals. This strategy was chosen, as waiting list survival analyses may not be reliable beyond 1-year follow-up.[4]

The Modification of Diet in Renal Disease (MDRD) equation was used to estimate glomerular filtration rate (eGFR) in the recipient.[5,6] The eGFR in donors was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[7]

Definitions

In this study, EGL was defined as graft loss within 90 days after transplantation. Kidney transplant recipients who died within 90 days with a functioning kidney graft were not considered as EGL recipients. Recipients without EGL after their first kidney transplant procedure were considered the reference population. For ischemic periods of the donor kidneys, the following definitions were used: The first warm ischemic time in kidneys donated after circulatory death (DCD) is the time following the no-touch period after circulatory arrest and asystole until cold flush-out in the donor is commenced; the cold ischemia time is defined as the time from start of cold flush-out until the start of the vascular anastomosis at time of implantation in the recipient; the graft anastomosis time is the time from organ removal from static cold storage or hypothermic machine perfusion to reperfusion in the recipient. Immunized patients are patients who have panel reactive antibody (PRA) ranges of ≥6% and <85%.[8] Highly immunized patients have PRAs of ≥85%.[8]

Statistical analysis

IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY) was used for statistical analysis. Differences in donor, recipient and transplant characteristics were analyzed using the Mann-Whitney rank test for nonparametric data, independent Student's *t* test for normal distributed data and the chi-square test for categorical data. A multivariate regression analysis was used to identify factors associated with EGL. Variables with a $p < 0.1$ in the univariate analysis were entered in the multivariate regression analysis. Cox proportional hazards analyses, adjusted for clinically relevant variables (recipient age and sex) and statistical relevant variables ($p < 0.1$ in the univariate analysis), were performed to evaluate differences in patient- and death-censored graft survival. Patient survival was calculated from the date of transplant to the date of death (event), and patients were censored on the last day of follow-up, which was October 17, 2018. Survival time was truncated at 3 months or 10 years. Graft survival was calculated from the date of transplant to the registered date of graft failure (event). Patients were censored at the time of patient's

death, or at the time of last day of follow-up. Kaplan-Meier survival curves were generated for all groups of interest. Results are represented as adjusted Hazard Ratio (aHR) for the patient and graft survival analyses, and as Odds Ratio (OR) for the multivariate regression analysis with the corresponding 95% Confidence Interval (CI). In this study, missing data–coded as *unknown* for categorical variables–were excluded from analyses. For variables of primary interest (EGL, cause of EGL, and short-term patient and graft survival) there were no missing values. The frequency of missing data for secondary variables is shown in Supplemental Table 1. To exclude a possible missing data-related bias, additional sensitivity analyses were performed for different timeframes. *P* values < 0.05 were considered statistically significant.

Results

An evaluation of EGL was conducted in a cohort of 10,307 deceased donor kidney transplants that were performed between January 1, 1990 and January 1, 2018 in The Netherlands. Of these procedures, 8,511 were primary transplant procedures. The observed incidence of EGL after a first kidney transplant was 8.2% (699/8,511). Recipients with EGL received grafts from slightly older donors and had longer first warm ischemic, cold ischemic, as well as graft anastomosis times (Table 1). The main reported causes of EGL were rejection (30.2%), primary non-function (25.0%), and thrombosis or infarction (20.3%) (Table 2).

Factors associated with EGL were explored using a multivariate regression analysis. Considering the procedural and potential biological differences between organs donated after brain death (DBD) and organs donated after circulatory death (DCD), these donor types were analyzed separately. Common risk factors for the development of EGL in both DBD and DCD transplant procedures were donor age, stroke as donor's cause of death, and graft anastomosis time (Table 3 and 4). Additional risk factors for the DCD transplant procedures were diabetes mellitus in the donor and the duration of first warm and cold ischemic time (Table 3). For the DBD grafts, donor's last serum creatinine, the number of years on dialysis before transplantation, and a PRA value ≥6% were found to further associate with EGL (Table 4). Donor characteristics, such as donor diabetes and cardiac arrest, are only registered from 2002 onward. As such, there is a high proportion of missing data (Supplemental Table 1). Additional sensitivity analyses of the multivariate models were performed for the 2002-2018 timeframe, which showed similar outcomes (Supplemental Table 2A and 2B). Of note, formal significance was lost for the associations between *donor age* and *stroke as cause of death* and EGL in the DCD group [p = 0.07 and 0.09, respectively] (Supplemental Table 2A).

Table 1. Descriptive characteristics of recipients with and without early graft loss after a first transplant procedure.

		EGL n = 699 (8.2%)	Non-EGL n = 7812 (91.8%)	p-value
Donor	Donor type (% DCD)	222 (31.8)	2394 (30.6)	0.541
	Age (years)	49.8 ± 15.6	46.7 ± 16.3	<0.001
	Sex (% male)	381 (54.5)	4228 (54.1)	0.845
	Height (cm)	173.0 [166.8–180.0]	175.0 [168.0–180.0]	0.007
	Weight (kg)	76.1 ± 17.6	75.5 ± 15.9	0.372
	BMI (kg/m^2)	25.6 ± 5.0	24.8 ± 4.2	0.001
	Last eGFR (CKD-EPI)	91.2 [69.6–106.1]	96.3 [75.6–111.1]	<0.001
	Cause of death			<0.001
	- Trauma	150 (21.5)	2333 (29.9)	
	- Stroke	422 (60.4)	3886 (49.7)	
	- Cardiac arrest	30 (4.3)	446 (5.7)	
	- Other	97 (13.9)	1147 (14.7)	
	Hypertension			0.002
	- Yes	157 (22.5)	1505 (19.3)	
	- No	321 (45.9)	4211 (53.9)	
	- Not registered[1]	221 (31.6)	2096 (26.8)	
	Diabetes			<0.001
	- Yes	36 (5.1)	243 (3.1)	
	- No	273 (39.1)	4001 (51.2)	
	- Not registered[2]	390 (55.8)	3568 (45.7)	
	Smoking			0.090
	- Yes	247 (35.3)	2870 (36.7)	
	- No	206 (29.5)	2826 (26.2)	
	- Not registered[1]	246 (35.2)	2116 (27.1)	
	Cardiac arrest			0.768
	- Yes	176 (25.2)	1957 (25.1)	
	- No	379 (54.2)	4334 (55.5)	
	- Not registered[2]	144 (20.6)	1512 (19.5)	

Table 1 continued

		EGL n = 699 (8.2%)	Non-EGL n = 7812 (91.8%)	p-value
Recipient	Age (years)	51.6 ± 14.2	51.6 ± 14.1	0.937
	Sex (% male)	409 (58.5)	4698 (60.1)	0.400
	Height (cm)	171.2 ± 10.5	171.4 ± 10.1	0.737
	Weight (kg)	76.8 ± 15.9	74.5 ± 15.0	0.001
	BMI (kg/m^2)	26.4 ± 4.8	25.3 ± 4.4	<0.001
	Cause of renal failure			0.050
	- Diabetes	57 (8.5)	740 (9.9)	
	- Hypertension	80 (11.9)	881 (11.8)	
	- Glomerulonephritis	84 (12.5)	785 (10.5)	
	- (Poly)cystic kidney disease	101 (15.1)	1184 (15.9)	
	- Pyelonephritis	57 (8.5)	620 (8.3)	
	- IgA nephropathy	21 (3.1)	412 (5.5)	
	- Chronic renal failure, etiology unknown	100 (14.9)	1197 (16.0)	
	- Other	171 (25.5)	1651 (22.1)	
	Pre-emptive			0.958
	- Yes	19 (2.7)	215 (2.8)	
	- No	679 (97.1)	7586 (97.1)	
	Time on dialysis (years)[1,3]	4.2 ± 2.4	3.8 ± 2.2	0.003
	Panel reactive antibodies			0.002
	- PRA <6%	601 (86.0)	7040 (90.1)	
	- PRA ≥6 and <85	89 (12.7)	707 (9.1)	
	- PRA ≥85	9 (1.3)	63 (0.8)	
	Mismatches			
	HLA-DR 0	291 (41.8)	3468 (44.5)	0.242
	1	368 (52.8)	3852 (49.5)	0.010
	2	38 (5.5)	465 (6.0)	<0.001
	HLA-A 0	209 (29.9)	2762 (35.4)	
	1	376 (53.9)	3947 (50.6)	
	2	113 (16.2)	1087 (13.9)	
	HLA-B 0	127 (18.2)	2017 (25.9)	
	1	413 (59.2)	4255 (54.6)	
	2	158 (22.6)	1524 (19.5)	
Transplant	First warm ischemic time in DCD grafts (min.)	20.0 [16.0 – 27.0]	17.0 [14.0 – 21.0]	<0.001
	Cold ischemic time (hours)	22.0 [16.7–27.3]	19.1 [14.0–24.5]	<0.001
	Graft anastomosis time (min.)	35.0 [28.0–45.0]	33.0 [26.0–40.0]	<0.001

BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; DCD, donation after circulatory death; eGFR, estimated glomerular filtration rate; EGL, early graft loss; HLA, human leukocyte antigen; min., minutes; PRA, panel reactive antibodies.

Data are presented as mean (± standard deviation), or as median [IQR], or as number (%).

[1] The variables hypertension, smoking and time on dialysis are consistently registered from 2000 onwards.

[2] The variables diabetes and cardiac arrest are consistently registered from 2002 onwards.

[3] Only applicable to non-pre-emptive recipients.

Table 2. Causes of early graft loss after first transplant procedures.

Causes of early graft loss	**n (%)** **Total n = 699**
Rejection	211 (30.2)
Primary non-function	175 (25.0)
Thrombosis or infarction	142 (20.3)
Technical or operative problems	96 (13.7)
Infection (graft and non-graft related)	12 (1.7)
Recurrent primary disease	10 (1.4)
Other	53 (7.6)

Data are presented as number (%).

Table 3. Multivariate analysis (Odds Ratio (95% CI)): risk factors associated with early graft loss after a first DCD transplant procedure.

		DCD
Donor	Age (years)	1.018 (1.003–1.034)*
	Height (cm)	0.987 (0.969–1.006)
	BMI (kg/m^2)	1.014 (0.978–1.050)
	Last creatinine (μmol/L)	1.005 (0.999–1.011)
	Hypertension	1.102 (0.715- 1.698)
	Diabetes	2.815 (1.537–5.155)**
	Cause of death	
	- Trauma	Reference
	- Stroke	1.704 (1.051 – 2.764)*
	- Cardiac arrest	0.782 (0.380 – 1.609)
	- Other	1.930 (1.094 – 3.405)*
Recipient	Cause of renal failure	
	- (Poly)cystic kidney disease	Reference
	- Diabetes	0.937 (0.464 – 1.894)
	- Hypertension	1.176 (0.602 – 2.297)
	- Glomerulonephritis	1.179 (0.543 – 2.563)
	- Pyelonephritis	0.979 (0.394 – 2.436)
	- IgA nephropathy	0.409 (0.117 – 1.435)
	- Chronic renal failure, etiology unknown	0.979 (0.518 – 1.849)
	- Other	1.238 (0.689 – 2.224)
	PRA ≥6%	1.538 (0.640 – 3.698)
Transplant	First warm ischemic time (min.)	1.049 (1.023–1.076)**
	Cold ischemic time (hours)	1.049 (1.014–1.085)*
	Graft anastomosis time (min.)	1.026 (1.014–1.038)**
	Year of transplant procedure	0.962 (0.914–1.012)

BMI, body mass index; DCD, donation after circulatory death; IgA, immunoglobulin A; min., minutes; PRA, panel reactive antibodies.

Variables with a p <0.1 in the univariate analysis were entered. *p <0.05; ** p <0.005

Table 4. Multivariate analysis (Odds Ratio (95% CI)): risk factors associated with early graft loss after a first DBD transplant procedure.

		DBD
Donor	Age (years)	1.030 (1.011–1.051)**
	Height (cm)	0.978 (0.956 – 1.001)
	BMI (kg/m^2)	0.968 (0.917–1.021)
	Last creatinine (µmol/L)	1.008 (1.004–1.013)**
	Hypertension	0.985 (0.634–1.531)
	Cause of death:	
	- Trauma	Reference
	- Stroke	1.982 (1.065 – 3.688)*
	- Cardiac arrest	1.413 (0.428 – 4.665)
	- Other	1.017 (0.365 – 2.832)
Recipient	Weight (kg)	1.002 (0.979–1.026)
	BMI (kg/m^2)	1.020 (0.943–1.104)
	Cause of renal failure	
	- (Poly)cystic kidney disease	Reference
	- Diabetes	0.568 (0.249 – 1.297)
	- Hypertension	0.564 (0.265 – 1.198)
	- Glomerulonephritis	0.461 (0.150 – 1.421)
	- Pyelonephritis	0.861 (0.348 – 2.130)
	- IgA nephropathy	0.335 (0.075 – 1.492)
	- Chronic renal failure, etiology unknown	0.808 (0.417–1.566)
	- Other	1.034 (0.560 – 1.909)
	Time on dialysis (years)	1.127 (1.042 – 1.218)**
	PRA ≥6%	2.502 (1.346 – 4.652)**
Transplant	Mismatch HLA-DR	
	0	1.218 (0.747 – 1.985)
	1	1.346 (0.639–2.832)
	2	
	Mismatch HLA-A	
	0	1.108 (0.671–1.828)
	1	1.784 (0.966 – 3.294)
	2	
	Mismatch HLA-B	
	0	1.409 (0.756–2.627)
	1	1.392 (0.685 – 2.831)
	2	
	Cold ischemic time (hours)	1.019 (0.989–1.049)
	Graft anastomosis time (min.)	1.027 (1.014–1.040)**
	Year of transplant procedure	1.012 (0.967–1.060)

BMI, body mass index; DBD, donation after brain death; HLA, human leukocyte antigen; IgA, immunoglobulin A; min., minutes; PRA, panel reactive antibodies.

Variables with a p <0.1 in the univariate analysis were entered. *p <0.05; ** p <0.005

The consequences of EGL on mortality, relisting, re-transplantation and outcomes of re-transplantation are summarized in Figure 1. EGL was associated with a significant increase in short-term mortality and compromised long-term patient survival. In fact, 30-day and 90-day mortality rates of the recipients with EGL were 5.2% and 11.7%, respectively (Figure 1), compared with 0.8% and 1.7% in the reference population (i.e., recipients without EGL after their first kidney transplant procedure). This survival disadvantage persisted in the long term, with a significantly higher 10-year mortality risk (early-death censored) among the EGL recipients [aHR 1.68 (95% CI: 1.33-2.13); $p < 0.001$] (Figure 2). Short-term and long-term patient survival after rejection-related or nonrejection-related EGL was similar (Supplemental Figure 1). Short-term and long-term causes of death are summarized in Table 5. The main causes of death were cardiovascular- and infection-related. The profound impact of EGL on patients experiencing EGL is clearly illustrated by a time of benefit of 5 years when compared with the estimated outcomes for patients on the waiting list (Figure 3).

Nearly three-quarters of the EGL recipients who survived 90 days after transplantation were relisted for re-transplantation, one-quarter did not return to the waiting list (Figure 1). The non-relisted recipients were approximately 10 years older than the relisted patients

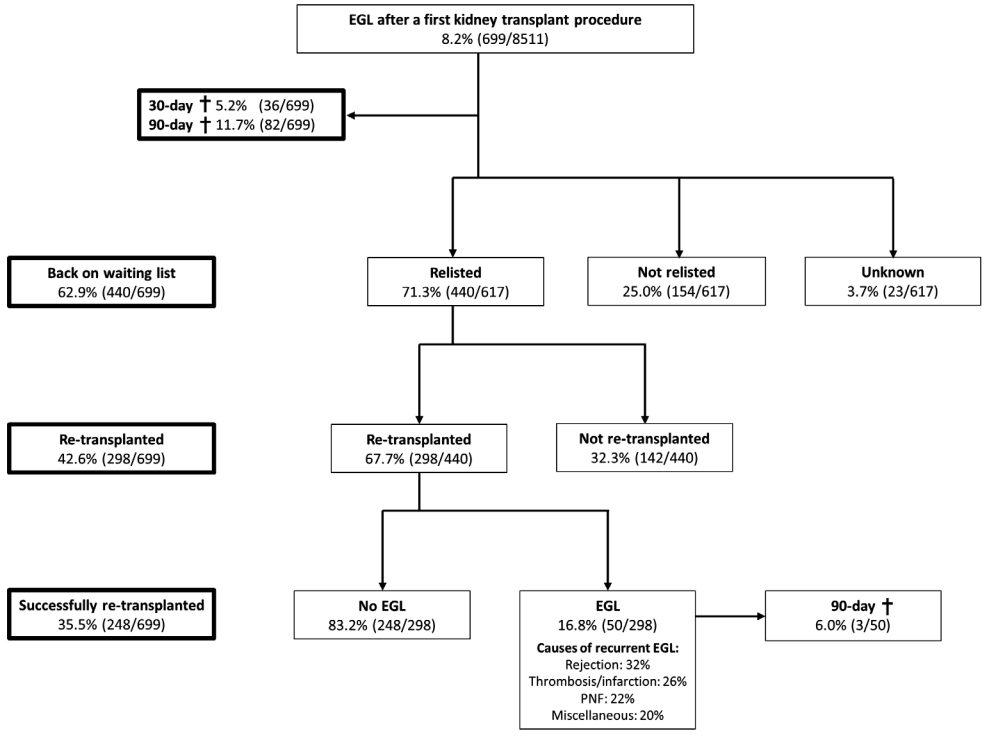

Figure 1. The consequences of early graft loss on mortality, relisting, re-transplantation and outcomes of re-transplantation. *EGL, early graft loss; PNF, primary non-function.*

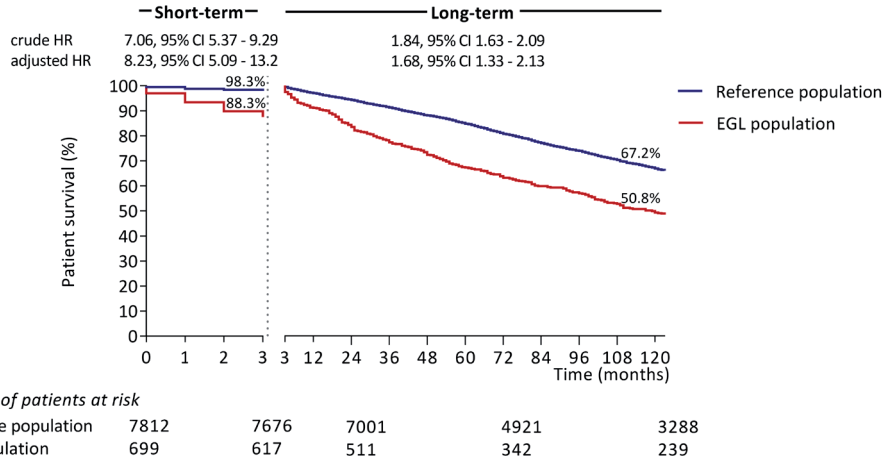

Figure 2. Landmark analysis showing the short-term and long-term patient survival rates.
Reference population: recipients without early graft loss after a first kidney transplant procedure. EGL population: recipients with early graft loss after a first kidney transplant procedure.
The model adjusted for clinical relevant variables and variables with a p <0.1 in the univariate analysis. The short-term was adjusted for: donor's cause of death, donor diabetes, recipient age, sex, height, BMI, cause of renal failure, pre-emptive, time on dialysis, mismatch HLA-DR, cold ischemic time and year of transplant procedure. The long-term was adjusted for: donor's cause of death, diabetes, hypertension, cardiac arrest, age, sex, height, BMI, last creatinine, and recipient age, sex, weight, BMI, cause of renal failure, pre-emptive, time on dialysis, PRA, mismatch HLA-DR, -A and -B, graft anastomosis time and year of transplant procedure.
CI, confidence interval; EGL, early graft loss; HR, hazard ratio.

Table 5. Causes of death among primary transplant recipients with early graft loss.

	Short-term mortality		**Long-term mortality**
	≤ 30 days	> 30 and ≤ 90 days	> 90 days and ≤ 10 years
Infectious	10 (27.8)	13 (28.3)	56 (20.2)
Hemorrhage	2 (5.6)	5 (10.9)	8 (2.9)
Cardiovascular	13 (36.1)	5 (10.9)	43 (15.5)
Cerebrovascular	1 (2.8)	1 (2.2)	13 (4.7)
Dialysis related[1]	-	-	27 (9.7)
Malignancy	-	1 (2.2)	14 (5.1)
Miscellaneous	8 (22.2)	8 (17.4)	12 (4.3)
Not determined	2 (5.6)	13 (28.3)	104 (37.5)
Total	36	46	277

Data are presented as number (%).
[1] Includes also patients that refused further dialysis treatment.

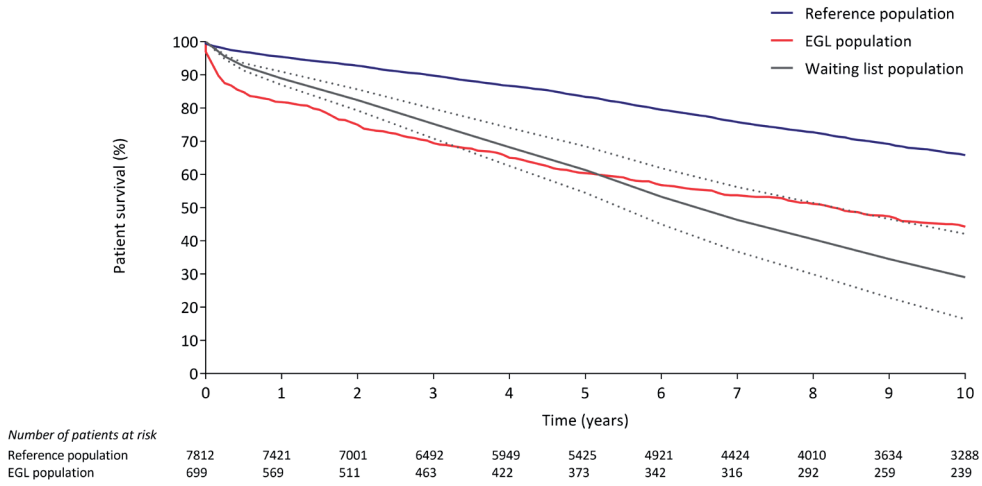

Figure 3. The impact of early graft loss on time to benefit for primary transplantations.
Reference population: recipients without early graft loss after a first kidney transplant procedure. EGL population: recipients with early graft loss after a first kidney transplant procedure. Waiting list population: simulated patient survival curve of active waitlisted (kidney only) patients in The Netherlands. The bottom and top dashed lines represent the corresponding 10th and 90th percentiles, respectively.

(Table 6). There were no indications that non-relisted patients were longer on dialysis before the initial transplant procedure (Table 6). Of the relisted patients, two-thirds were subsequently re-transplanted resulting in an actual re-transplantation rate of 42.6% (Figure 1). The re-transplanted recipients were slightly younger compared with relisted not-re-transplanted recipients (mean age: 46.6 vs. 50.6 years, respectively) (Table 6). The proportion of immunized (45%) and highly immunized (24%) patients was equal in both groups (Table 6).

The analysis for re-transplantation showed a clear compromised outcome, with a doubled EGL incidence (16.8% vs. 8.2%; Figure 1). Among the re-transplanted patients, 83.2% were successfully re-transplanted (i.e., recipients without EGL after re-transplantation), resulting in an overall successful re-transplantation rate of 35.5% (Figure 1). For those successfully re-transplanted, 3-month and 1-year graft function (eGFR) was equal compared with the reference group (p = 0.33 and 0.26, respectively). Although long-term graft survival after re-transplantation was inferior [crude HR 1.47; (95% CI: 1.11-1.94)], significance was lost after adjustment for potential confounders [aHR 1.06; (95% CI: 0.62-1.81)] (Figure 4). Subgroup analysis of long-term graft survival after rejection-related EGL showed a similar pattern; crude HR 2.42; (95% CI 1.59-3.70) and aHR 1.71; (95% CI 0.67-4.33) (Supplemental Figure 2).

Table 6. Comparison analyses of recipient characteristics.

	Recipients with early graft loss after a first kidney transplant procedure.								Reference population n = 7812	
	Patients died ≤90 days after transplantation n = 82		Non-relisted n = 154		Relisted, not re-transplanted n = 142		Relisted, and re-transplanted n = 298			
Age (years)	57.3 ± 11.6 60.0 [50.0–65.0]		57.5 ± 12.6 60.0 [50.8–68.0]		50.6 ± 13.6 53.5 [42.5–60.0]		46.6 ± 14.2 48.0 [36.0–58.0]		51.6 ± 14.1 54.0 [43.0–63.0]	
Sex (% male)	50 (61)		82 (53)		81 (57)		183 (61)		4698 (60.1)	
BMI (kg/m^2)	26.8 ± 4.8		27.2 ± 4.9		26.6 ± 4.8		25.7 ± 4.4		24.8 ± 4.2	
Pre-emptive primary transplantation (% yes)	0		6 (4)		5 (4)		8 (3)		215 (2.8)	
Prior dialysis time before primary transplantation (years)	4.8 ± 2.3 4.6 [3.2–6.2]		4.1 ± 2.0 3.8 [2.7–5.2]		4.4 ± 2.7 4.0 [2.3–5.8]		4.0 ± 2.0 4.0 [2.6–5.1]		3.8 ± 2.2 3.6 [2.3–5.0]	
Panel reactive antibodies	n[1]	%	n[1]	%	n[2]	%	n[2]	%	n[1]	%
- PRA > 5 and < 85%	5	6	26	17	64	45	135	45	707	9
- PRA ≥ 85%	0	0	2	1	34	24	71	24	63	1
Cause of renal failure	n	%	n	%	n	%	n	%	n	%
- Chronic renal failure, etiology unknown	9	11	27	18	22	15	39	13	1197	15
- Diabetes Mellitus	12	15	16	10	12	8	15	5	740	10
- Glomerulonephritis	6	7	14	9	14	10	45	15	785	10
- Hypertension	16	20	20	13	12	8	30	10	881	11
- IgA nephropathy	1	1	3	2	4	3	13	4	412	5
- (Poly)cystic kidney disease	11	13	22	14	20	14	47	16	1184	15
- Pyelonephritis	4	5	8	5	5	4	38	13	620	8
- Miscellaneous	20	24	38	25	42	30	66	22	1651	21
- Not specified	3	4	6	4	11	8	5	2	342	4

BMI, body mass index; IgA, immunoglobulin A; PRA, panel reactive antibodies.

Data are presented as mean (± standard deviation), or as median [IQR], or as number (%).

[1] Most recent registered PRA percentage before the primary transplant procedure.

[2] Maximum PRA percentage registered after primary transplant procedure.

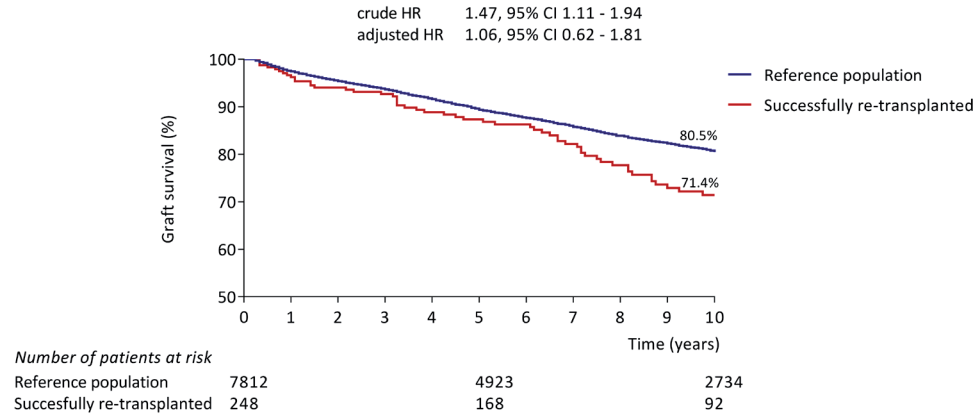

Figure 4. Death censored 10-year graft survival.
Reference population: recipients without early graft loss after a first kidney transplant procedure. Successfully re-transplanted: recipients with early graft loss after a first transplant procedure, and without early graft loss after a re-transplantation.
The model adjusted for clinical relevant variables and variables with a p <0.1 in the univariate analysis: donor type, donor age, sex, height, BMI, last creatinine, cause of death, hypertension, diabetes, recipient age, sex, cause of renal failure, PRA, mismatch HLA-A and -B, cold ischemic time, graft anastomosis time and year of transplant procedure.
CI, confidence interval; HR, hazard ratio.

Evaluation of a possible time effect showed a clear decrease in incidence of EGL over time ($p < 0.001$), yet the consequences of EGL were not influenced by time (Supplemental Table 3).

In the light of the profound impact of EGL, the question arises as to whether a more strict policy with regard to donor pool quality by only accepting grafts with a minimal chance of EGL (and thus longer waiting lists) outweighs waiting list mortality. Data for the Dutch cohort studied herein allowed for the evaluation of the impact of the 8.2% a priori risk of EGL on recipient survival. Figure 5 shows that the 8.2% EGL-risk for the Dutch donor pool minimally affects recipient survival [aHR (0% EGL is reference): 1.15 (95% CI: 0.99-1.34); $p = 0.07$], and that the consequences associated with a 8.2% EGL risk clearly outweighs simulated waiting-list mortality.

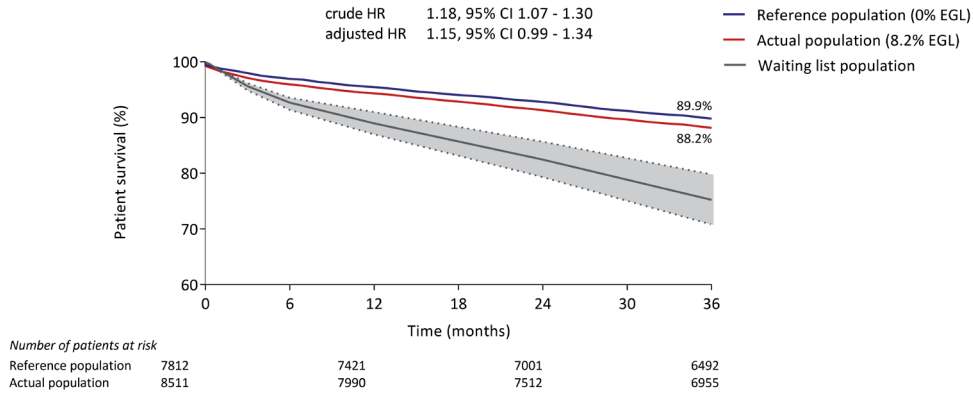

Figure 5. The impact of an 8.2% early graft loss incidence on 3-year patient survival following primary transplantation.

Reference population (0% EGL): patient survival for those successfully transplanted (EGL excluded). Actual population (8.2% EGL): Patient survival for a cohort with an 8.2% incidence of EGL. Waiting list population: simulated patient survival curve of active waitlisted (kidney only) patients in The Netherlands. The bottom and top dashed lines represent the corresponding 10th and 90th percentiles, respectively.

The 3-year mortality risk for a population with a 8.2% EGL risk was estimated in a Cox proportional hazards model (actual population versus reference population) that adjusted for clinical relevant variables and variables with a p <0.1 in the univariate analysis: donor age, sex, height, BMI, cause of death, hypertension, smoking, cardiac arrest, and recipient age, sex, height, BMI, cause of renal failure, time on dialysis, PRA, mismatch HLA-DR, -A and -B and graft anastomosis time.

CI, confidence interval; HR, hazard ratio.

Discussion

This nationwide evaluation characterizes EGL as a medical catastrophe that associates with a substantial short-term recipient mortality and poor long-term outcomes.

The profound benefits of kidney transplantation over dialysis on patient survival, quality of life, and costs—along with an aging population—have resulted in accruing waiting lists and increased waiting list associated mortality.[9-12] Attempts to expand the donor pool come with higher incidences of delayed graft function (DGF), inferior function at 12 months, and (early) graft failure. Yet, although DGF is often regarded as a major impediment, recent cohort studies show that for DCD grafts, DGF does not impair long-term graft and patient survival and, consequently, that DGF in DCD grafts should be regarded an acceptable complication.[13-15] Graft failure, on the other hand, is considered a disastrous complication of kidney transplantation. As such, an increased risk of graft failure—and particularly early graft failure— should be considered the primary impediment for a liberal use of deceased donor kidneys.

Yet, although several cohort studies show that EGL has a negative impact on patient survival,[16-19] only 2 single-center, medium-sized (50 and 109 EGL cases, respectively) studies from the United Kingdom and Ireland systematically evaluated the further wide-ranging consequences of EGL.[1,2] However, these studies were underpowered. Moreover, EGL was defined as graft nephrectomy or loss of transplant function within 30 days after transplantation.[1,2] Although 30-day outcomes are generally used as primary outcomes for surgical complications, this time point may not accurately reflect the actual incidence of EGL. DGF may extend beyond 30 days,[20-22] and a considerable number of graft losses may only be diagnosed after 30 days.[1,23] As a consequence, the 30-day time frame is too short to justify a robust medical decision, as the clinical diagnosis of EGL may be made at a time point beyond 30 days. In this context, we considered the 90-day time frame more appropriate, as regulations within Eurotransplant allow recipients with graft failure within 90 days to retain their initial pre-transplantation waiting times in case of relisting. Consequently, the 90-day time point hallmarks a strong external impetus for clinical decision-making with respect to the diagnosis of EGL. As such, it was decided to define EGL as functional graft loss within 90 days after transplantation. Based on this definition, we identified almost 700 recipients with EGL after their first kidney transplant in the national registry and performed a systematic, in-depth evaluation of the overall impact of EGL.

Although the overall incidence of EGL (8.2%) in this evaluation suggests a 2.5-fold higher incidence than in the United States (3.4%),[17] it is important to bear in mind that this is partly due to a time-dependent effect with higher incidences of EGL in the earlier years.[1] In fact, the incidence of EGL in The Netherlands for the corresponding time period (i.e., 2011 to 2015) is 5.4%. The moderately higher incidence presumably reflects a more liberal attitude toward accepting DCD kidneys[24] and the fact that the donors (for the 2011–2015 timeframe) are approximately 16 years older in The Netherlands than in the United States.[17] It has to be noted that, although multivariate analyses mainly identified donor characteristics as risk factors for the development of EGL (Tables 3 and 4), the models only cover 14% (for DCD) and 13% (for DBD) of the variation by the explanatory variables as estimated by Nagelkerke R^2.[25] This implies that the majority of causative factors are not captured by the current database and that, apart from donor and procedural factors, recipient factors also associate with the development of EGL. A notable aspect is the observed significant risk of EGL in recipients who received grafts from diabetic DCD donors (Table 3). Although this alarming finding obviously requires external confirmation, it calls for restraint in use of these donors. Although of interest, the available registry data did not allow further exploration of the negative impact of diabetes in DCD donors. One possible explanation for the phenomenon is that donor diabetes interferes with superior resilience responses observed in DCD donor kidneys.[26]

This study confirms the findings of earlier studies with regard to the profound impact of EGL on patient survival. Whereas the Dutch registry data indicate a 30- and 90-day mortality rate of 1% and 2%, respectively, in the non-EGL group, an almost 7-fold higher incidence was observed in the EGL group. Although this high mortality may obviously be a direct consequence of EGL,[2,27] the EGL-associated 90-day mortality also includes EGL that results from a recipient's death. To be more specific, grafts in patients who died perioperatively are denied the opportunity to regain their function. Although the increased mortality may directly be related to surgical complications,[2,28] it presumably also involves accumulation of recipient-related risk factors such as a higher age, poor (cardio)vascular status, and/or an increased frailty.[29]

Apart from the immediate impact of EGL on mortality, the data indicate far-reaching, long-term consequences. Based on data from the Eurotransplant registry, 25% of the recipients with EGL were not relisted for re-transplantation. Reasons for not relisting are not captured in the Netherlands Organ Transplant Registry (NOTR) and Eurotransplant registry, and considerations are generally missing from patient records. Specified motivations for not relisting included patients' preferences or recipient's health status such as overall functional status or frailty, cardiovascular status or vascular condition.[30] These aspects are reflected by the approximately 10-year older age of the non-relisted versus the relisted patients (Table 6).

One-third of the patients relisted after EGL did not undergo re-transplantation. Although this might be a slight overestimation, owing to some recipients with more recent EGL may be still awaiting re-transplantation, the majority of recipients is most likely removed from the waiting list because of worsening clinical condition or death. Although no specific information was available to what extent sensitization determines eligibility for relisting, sensitization status did not seem to influence the probability of re-transplantation among the relisted patients, as the number of immunized and highly immunized patients was equally distributed between the retransplanted and not–re-transplanted groups.

Only 43% of the EGL patients underwent retransplantation. Outcomes for these re-transplantations are inferior compared with those of first kidney transplants. Noticeably, re-transplantation was associated with a doubled incidence of EGL (16.8% vs. 8.2% for primary transplantations and 9.1% for re-transplantations following late graft loss). This presumably reflects the convergence of risk factors within the individual patient, indicating that patients with EGL after a first transplant should be considered a high-risk recipient. This increased risk of recurrent EGL will obviously compromise the time to benefit for re-transplantations,[30-35] an aspect that should be accounted for when considering re-transplantation.

In the light of the profound impact of EGL, the question arises whether a risk of EGL outweighs the waiting list mortality. Although such an analysis is prone to confounding by indication,[4] the cohort data allowed us to estimate the impact of the 8.2% a priori risk of EGL at the population level. It was concluded that, despite the profound impact of EGL for the individual recipient, the 8.2% incidence affected the population risk minimally. On this basis, it can be concluded that an optimal trade-off between the risk of EGL and waiting list mortality is beyond an a priori EGL risk of 8.2% and that a policy aimed at minimizing incident EGL will lead to avoidable deaths as a result of longer waiting list times.

A further question is whether patients who sustained EGL would have been better off with remaining on the waiting list. Although the data herein imply a time to benefit of 5 years after EGL, this poor outcome is actually a reflection of asymmetrical outcomes, with a strikingly high 6-month mortality for a subgroup of EGL recipients but favorable outcomes for EGL recipients surviving 6 months. One could speculate that the early mortality affects a subgroup of vulnerable recipients who were, in retrospect, better off staying on dialysis. In this context, accurate prediction tools aimed at identifying patients at risk of early death after EGL are an unmet medical need.[4]

Our study has some limitations, as this is a registry-based study. Although the NOTR is a mandatory registry for all 8 Dutch transplant centers, and several quality checks are performed, there are remaining missing data and registration errors. In addition, recipient factors of potential relevance, such as frailty, comorbidities, and cardiac and vascular state, are not included in the registry. Another limitation is that the vast majority of the patients in this evaluation are Caucasian. Given the profound impact of race on transplant outcomes, conclusions may not fully apply to non-Caucasians.[36] Finally, conclusions are influenced by medical decision-making; kidneys with an anticipated risk of EGL are often declined before organ procurement (selection bias by allocation), and only a selective group of patients are considered eligible for relisting and re-transplantation (selection bias by indication).

In conclusion, the results in this nationwide study show that EGL after kidney transplantation is associated with significant detrimental consequences. These consequences include profound short-term and long-term mortality rates, a reduced chance of relisting and re-transplantation, and—for those re-transplanted—an increased risk of recurrent EGL. Although the development of EGL and the associated poor outcomes are generally attributed to the use of suboptimal kidney grafts, the data in this study also imply convergence of recipient risk factors in patients with EGL. As such, these recipient factors should specifically be accounted for when estimating the optimal trade-off at which the impact of EGL is balanced by maximizing the donor pool–size.

With respect to the donor and procedural factors, the multivariate analyses performed show that, after the medical decision to accept the graft for donation, traditional risk factors minimally associate with incident EGL. Hence, there is an urgent need for complementary risk-assessment tools, such as biomarkers or ex vivo functional organ assessment, and possibly more extended risk prediction models.

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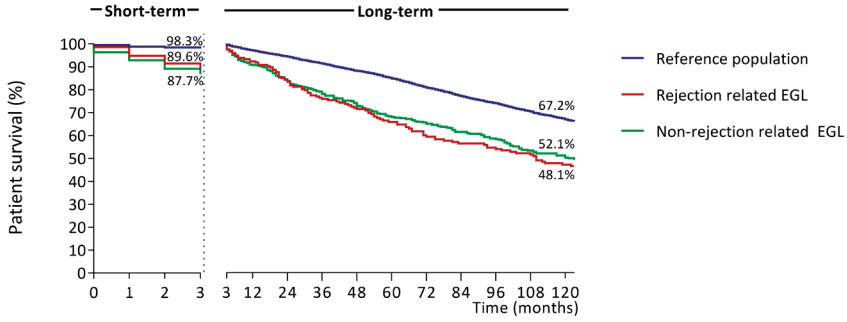
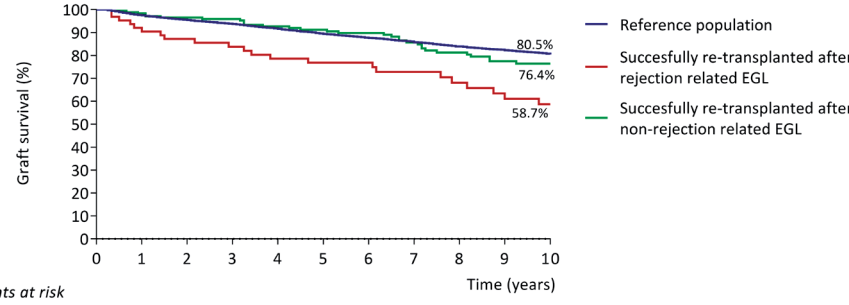
Supplemental Data



Number of patients at risk

Reference population	7812	7676	7001	4921	3288
Rejection related EGL	211	189	158	101	75
Non-rejection related EGL	488	428	353	241	164

Supplemental Figure 1. Landmark analysis of short-term and long-term patient survival following rejection resp. nonrejection-related EGL.
Reference population: recipients without EGL after a first kidney transplant procedure. Rejection related EGL: recipients with rejection related EGL after a first kidney transplant procedure. Non-rejection related EGL: recipients with non-rejection related EGL after a first kidney transplant procedure.



Number of patients at risk

Reference population	7812	4923	2734
Rejection related EGL	67	43	23
Non-rejection related EGL	181	125	69

Supplemental Figure 2. Death censored 10-year graft survival following successful re-transplantation after primary rejection resp. nonrejection-related EGL.
Reference population: recipients without EGL after a first kidney transplant procedure.
Crude HRs (95% CI) rejection related and non-rejection related EGL versus reference population: 2.42 (1.59-3.70) and 1.15 (0.80-1.64), respectively.
Adjusted HRs (95% CI) rejection related and non-rejection related EGL versus reference population: 1.71 (0.67-4.33) and 0.85 (0.45-1.62), respectively.
CI, confidence interval; HR, hazard ratio.

Supplemental Table 1. Proportion of missing data.

		From 1990 to 2018		From 2002 to 2018	
		Number of missing values	% Missing	Number of missing values	% Missing
Donor	Donor type (DBD/DCD)	0	0	0	0
	Age	0	0	0	0
	Sex	0	0	0	0
	Height	558	6.6	0	0
	Weight	537	6.3	2	<0.01
	BMI	567	6.7	2	<0.01
	Last creatinine	193	2.3	84	1.7
	Cause of death	0	0	0	0
	Hypertension	2317	27.2	425	8.5
	Diabetes	3958	46.5	443	8.9
	Smoking	2362	27.8	311	6.2
	Cardiac arrest	1665	19.6	374	7.5
Recipient	Age	0	0	0	0
	Sex	0	0	0	0
	Height	1713	20.1	208	4.2
	Weight	1677	19.7	225	4.5
	BMI	1903	22.4	321	6.4
	Cause of renal failure	370	4.3	279	5.6
	Pre-emptive	12	<0.01	0	0
	Time on dialysis[1]	2968	35.9	55	1.1
	PRA	2	<0.01	0	0
	Mismatch HLA-DR	29	<0.01	29	<0.01
	Mismatch HLA-A	17	<0.01	17	<0.01
	Mismatch HLA-B	17	<0.01	17	<0.01
Transplant	First warm ischemic time[2]	118	4.5	106	4.5
	Cold ischemic time	509	6.0	405	8.1
	Graft anastomosis time	593	7.0	473	9.5

BMI, body mass index; DBD, donation after brain death, DCD, donation after circulatory death; HLA, human leukocyte antigen; PRA, panel reactive antibodies.

[1] Only applicable to non-pre-emptive recipients.

[2] Only applicable to DCD kidneys.

Supplemental Table 2A. Multivariate sensitivity analysis including data from 2002 to 2018 (Odds Ratio (95% CI)): risk factors associated with early graft loss after a first DCD transplant procedure.

		DCD
Donor	Age (years)	1.014 (0.999 – 1.030)
	BMI (kg/m^2)	1.009 (0.973 – 1.046)
	Hypertension	1.136 (0.731 – 1.765)
	Diabetes	2.567 (1.345 – 4.899)**
	Smoking	1.400 (0.958 – 2.046)
	Cause of death	
	- Trauma	Reference
	- Stroke	1.530 (0.936 – 2.500)
	- Cardiac arrest	0.738 (0.357 – 1.529)
	- Other	1.972 (1.113 – 3.494)*
Recipient	Cause of renal failure	
	- (Poly)cystic kidney disease	Reference
	- Diabetes	0.967 (0.479 – 1.951)
	- Hypertension	1.133 (0.571 – 2.247)
	- Glomerulonephritis	1.090 (0.495 – 2.401)
	- Pyelonephritis	1.115 (0.465 – 2.673)
	- IgA nephropathy	0.450 (0.129 – 1.570)
	- Chronic renal failure, etiology unknown	0.939 (0.494 – 1.783)
	- Other	1.316 (0.735 – 2.357)
	PRA ≥6%	1.553 (0.670 – 3.595)
Transplant	First warm ischemic time (min.)	1.050 (1.024 – 1.077)**
	Cold ischemic time (hours)	1.058 (1.023 – 1.094)**
	Graft anastomosis time (min.)	1.027 (1.015 – 1.040)**
	Year of transplant procedure	0.960 (0.913 – 1.011)

BMI, body mass index; DCD, donation after circulatory death; IgA, immunoglobulin A; min., minutes; PRA, panel reactive antibodies.

Variables with a p <0.1 in the univariate analysis were entered. *p <0.05; ** p <0.005

Supplemental Table 2B. Multivariate sensitivity analysis including data from 2002 to 2018 (Odds Ratio (95% CI)): risk factors associated with early graft loss after a first DBD transplant procedure.

		DBD
Donor	Age (years)	1.042 (1.022 – 1.062)**
	BMI (kg/m^2)	1.004 (0.959 – 1.051)
	Last creatinine (µmol/L)	1.003 (1.003 – 1.010)**
	Cause of death:	
	- Trauma	Reference
	- Stroke	2.043 (1.080 – 3.866)*
	- Cardiac arrest	1.813 (0.635 – 5.178)
	- Other	1.465 (0.625 – 3.435)
Recipient	Weight (kg)	0.993 (0.972 – 1.015)
	BMI (kg/m^2)	1.077 (1.004 – 1.156)
	Time on dialysis (years)	1.146 (1.059 – 1.242)**
	PRA ≥6%	2.053 (1.112 – 3.791)*
Transplant	Mismatch HLA-DR	
	0	Reference
	1	1.598 (0.986 – 2.589)
	2	1.387 (0.670 – 2.873)
	Mismatch HLA-A	
	0	Reference
	1	1.051 (0.655 – 1.686)
	2	1.306 (0.707 – 2.413)
	Mismatch HLA-B	
	0	Reference
	1	1.291 (0.704 – 2.365)
	2	1.341 (0.691 – 2.602)
	Cold ischemic time (hours)	1.015 (0.987 – 1.044)
	Graft anastomosis time (min.)	1.023 (1.011 – 1.035)**

BMI, body mass index; DBD, donation after brain death; HLA, human leukocyte antigen; min., minutes; PRA, panel reactive antibodies.
Variables with a p <0.1 in the univariate analysis were entered. *p <0.05; ** p <0.005

Supplemental Table 3. Time-related changes in incidence and consequences of early graft loss.

Early graft loss and consequences	1990-2018 (full dataset)	1990-2004 (period 1)	2004-2018 (period 2)
% Early graft loss	8.2%	10.0%	6.6%[1]
% Recurrent early graft loss	16.8%	19.0%	12.8%[ns]
Short-term patient survival[2]			
- Crude HR	7.06 (5.37 – 9.29)	5.77 (4.04 – 8.23)	8.80 (5.73 – 13.5)
- Adjusted HR	8.23 (5.09 – 13.2)	6.25 (1.36 – 28.68)	8.76 (5.22 – 14.70)
Long-term patient survival[3]			
- Crude HR	1.84 (1.63 – 2.09)	1.84 (1.57 – 2.15)	1.88 (1.53 – 2.31)
- Adjusted HR	1.68 (1.33 – 2.13)	2.53 (1.33 – 4.81)	1.68 (1.30 – 2.18)
Long-term graft survival successfully re-transplanted patients[4]	1.47 (1.11 – 1.94)	1.42 (0.99 – 2.03)	1.53 (0.97 – 2.43)
- Crude HR	1.06 (0.62 – 1.81)	0.56 (0.13 – 2.41)	1.22 (0.68 – 2.16)
- Adjusted HR			

EGL, early graft loss; HR, hazard ratio; ns, not significant.

[1] $p < 0.001$

[2] EGL versus non-EGL recipients after a primary transplant procedure. Factors adjusted for are provided in the legend to Figure 2.

[3] EGL versus non-EGL recipients after a primary transplant procedure. Factors adjusted for are provided in the legend to Figure 2.

[4] Successfully re-transplanted recipients after a primary EGL transplant procedure versus non-EGL versus primary non-EGL recipients. Factors adjusted for are provided in the legend to Figure 3.

Chapter 3

Improving outcomes for donation after circulatory death kidney transplantation: Science of the times

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Abstract

The use of kidneys donated after circulatory death (DCD) remains controversial due to concerns with regard to high incidences of early graft loss, delayed graft function (DGF), and impaired graft survival. As these concerns are mainly based on data from historical cohorts, they are prone to time-related effects and may therefore not apply to the current timeframe. To assess the impact of time on outcomes, we performed a time-dependent comparative analysis of outcomes of DCD and donation after brain death (DBD) kidney transplantations.

Data of all 11,415 deceased-donor kidney transplantations performed in The Netherlands between 1990-2018 were collected. Based on the incidences of early graft loss, two eras were defined (1998-2008 [n=3,499] and 2008-2018 [n=3,781]), and potential time-related effects on outcomes evaluated. Multivariate analyses were applied to examine associations between donor type and outcomes. Interaction tests were used to explore presence of effect modification.

Results show clear time-related effects on posttransplant outcomes. The 1998-2008 interval showed compromised outcomes for DCD procedures (higher incidences of DGF and early graft loss, impaired 1-year renal function, and inferior graft survival), whereas DBD and DCD outcome equivalence was observed for the 2008-2018 interval. This occurred despite persistently high incidences of DGF in DCD grafts, and more adverse recipient and donor risk profiles (recipients were 6 years older and the KDRI increased from 1.23 to 1.39 and from 1.35 to 1.49 for DBD and DCD donors). In contrast, the median cold ischemic period decreased from 20 to 15 hours.

This national study shows major improvements in outcomes of transplanted DCD kidneys over time. The time-dependent shift underpins that kidney transplantation has come of age and DCD results are nowadays comparable to DBD transplants. It also calls for careful interpretation of conclusions based on historical cohorts, and emphasises that retrospective studies should correct for time-related effects.

Introduction

In the past decades, organs retrieved from donation after brain death (DBD) donors have provided the majority of solid organ transplants globally. Due to the medical success of transplantation as an effective therapy for patients with end stage organ failure, the increased need of donor organs created a persistent shortage which has resulted in the death of many patients while waiting for a transplant.

For many years now, kidneys donated after circulatory death (DCD) have been proposed as an effective means of addressing this severe organ shortage.[1,2] Despite emerging reports indicating that mid-term and long-term outcomes of DCD procedures are better than commonly thought,[3-5] only some countries have fully embraced this opportunity.[6] For various reasons, others have been reluctant or even outspoken adverse towards the introduction of a controlled DCD programme that could alleviate the shortage and save many lives within a healthcare system.[7-8] While for some countries reasons to not or only slowly allow DCD programmes relate to ethical issues, legal restrictions or logistical concerns,[9] for the majority of countries the reticent attitude generally reflects medical concerns that are based on reported high incidences of early graft loss, delayed graft function (DGF), and an assumed impaired graft survival for DCD kidneys.

Since the concerns regarding the inferior DCD outcomes are mainly based on historical analyses, they are prone to time-related effects as time-varying confounding and effect modification by time.[10,11] Time-varying confounding is the phenomenon that the values of confounding variables, such as donor and recipient age, change over time.[10] Effect modification by time occurs when the effect of donor type on outcomes is modified by time (e.g. due to changes in procedural characteristics and/or medical decision-making over time).[11] Therefore, assumptions as regards inferior outcomes of DCD procedures may not apply anymore to our current timeframe.

To test whether conclusions with regard to the outcomes of DCD kidney transplant procedures are influenced by time, and to objectify the current results achieved when utilising DCD donor kidneys, we performed a longitudinal time-dependent comparative analysis of the outcomes of DBD and DCD kidney transplant procedures performed in The Netherlands, as country with a longstanding tradition of the use of DCD donor kidneys.

Materials and methods

Patient population

This national outcome evaluation was approved by the Ethics Committee of the Leiden University Medical Center, and the clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration

of Istanbul on Organ Trafficking and Transplant Tourism'. Data was fully anonymized prior to access and analysis.
In this study, we collected data of all 11,415 deceased-donor kidney transplant procedures performed in The Netherlands between 1990 and 2018. Combined organ procedures (n = 635), procedures with grafts donated after uncontrolled circulatory death (i.e. Maastricht Category I: dead on arrival and II: unsuccessful resuscitation) (n = 212), and procedures in recipients younger than 12 years old (n = 261) were excluded.
To explore a possible time-related effect, the incidence of early graft loss was mapped for the years 1990 to 2018. In this analysis, only primary kidney transplant procedures (n=8,511) were included since early graft loss after re-transplantation is potentially interfered with accumulation of recipient-related risk factors.[12] Based on the early graft loss incidences, two timeframes were defined for the time-dependent comparative analysis. This analysis included all (primary transplantations and re-transplantations) transplantations performed between 1998-2008 (n=3,499) and 2008-2018 (n=3,781).
Data was retrieved from the Dutch National Organ Transplant Registry, which is a mandatory registry that contains granular data of all eight Dutch kidney transplant centres.

Definitions

Early graft loss was defined as graft loss within 90 days after transplantation. Patients who died within 90 days after transplantation with a functioning graft were not considered as early graft loss recipients. DGF was defined as the need for dialysis in the first postoperative week(s). The Modification of Diet in Renal Disease (MDRD) equation was used to estimate the glomerular filtration rate (eGFR) in the recipient. The non-scaled, donor-only version of the Kidney Donor Risk Index (KDRI) was calculated as described by Rao et al..[13] The following definitions were used for ischemic periods of the donor kidneys. The first warm ischemic period is the time following the no touch period after circulatory arrest and asystole in the DCD donor, until cold flush-out in the donor is commenced. The cold ischemic period is the time from start of cold flush-out until the start of the vascular anastomosis in the recipient. The graft anastomosis time is defined as the time from kidney removal from static cold storage or hypothermic machine perfusion until reperfusion in the recipient.

Data analysis

IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Comparisons between DBD and DCD procedures were performed using the independent t-test for normal-distributed data, the Mann-Whitney rank test for non-parametric data, and the Chi-Square test for categorical data.

The KDRI was reported to facilitate comparison of the Dutch donor cohort with that of other countries. However, in the Dutch National Organ Transplant Registry donor hypertension and diabetes–which are included in the KDRI–are only registered from 2000 respectively 2002 onwards. As such, there was a high proportion of missing data for the 1998-2018 interval (26.6% for diabetes and 10.0% for hypertension) and multiple imputation of missing data of variables included in the KDRI was applied.
Logistic and linear regression analyses were used to examine the association between outcomes (DGF, early graft loss and 1-year eGFR) and donor type. Cox proportional hazards analyses were performed to evaluate differences in patient survival and death censored graft survival. All the multivariate models were adjusted for variables statistically relevant (p-value <0.10) in the univariate analysis (Supplemental Table 1). To avoid overcorrection the KDRI was not included in the multivariate models (as the KDRI also comprises donor age and donor type which are already included separately in the univariate and multivariate analyses). Also the type of preservation solution was not included as the inter-relationship between variables (the selection of preservation solution depends on donor type) would substantially impact the validity of the model. Results are represented as beta coefficient (β), Odds Ratio (OR) or Hazard Ratio (HR) with the corresponding 95% Confidence Interval (CI).
An interaction (Wald) test was used to explore the presence of effect modification by time. In other words, to test whether the effect of donor type on outcomes is modified by time. To specifically determine the association (R^2) between KDRI and 5-year graft survival, logistic regression analysis was performed using the data from 2002 to 2018 (non-imputed). P-values <0.05 were considered statistically significant.

Results

To explore a possible time-dependent effect on transplant outcomes, we mapped the incidence of early graft loss, as an unambiguous outcome parameter, for the years 1990 to 2018 in The Netherlands (Figure 1). Analyses of 5,895 DBD and 2,616 DCD primary kidney transplants performed in this period indicated clear time-related effects with 1998 (DBD) and 2008 (DCD) as clear transition years, after which the incidence of early graft loss dropped and stabilized at an incidence of approximately 6% (Figure 1).

Based on the transition years for early graft loss, two timeframes (1998-2008 and 2008-2018) were defined, and the outcomes of all 7,280 DBD and DCD procedures performed in these periods were compared accordingly (Table 1 and 2). This comparison showed a marked increase in donor and recipient age, and in KDRI over time: donors and recipients were respectively 6.5 and 6 years older in the recent (2008-2018) timeframe and the KDRI increased from 1.23 to 1.39 for DBD donors and from 1.35 to 1.49 for DCD

donors (Table 1). Although the KDRI for this Dutch cohort was significantly associated with 5-year graft survival ($p<0.001$), the Nagelkerke R^2 for the KDRI and 5-year graft loss was only 1.7% suggesting a limited impact of donor characteristics on graft survival. In contrast to the increase in donor and recipient age over time, there was a clear decrease in the cold ischemic period from a mean value of 20 hours in the 1998-2008 era to approximately 15 hours in the 2008-2018 era (Table 1). Also, the proportion of procedures with excessive cold ischemic periods (>24 hours) substantially decreased over time (from 25.7% to 7.8% and from 20.1% to 3.2% for DBD respectively DCD donors).
Furthermore, the type of preservation solution changed over time. Whereas histidine-tryptophan-ketoglutarate (HTK) solution was most common for DCD donor kidneys between 1998 and 2008 (86.1%), University of Wisconsin solution was most commonly used between 2008 and 2018 (57.8%) (Table 1).

The comparative outcome analysis showed clear time-related effects (Table 2). Whereas the 1998-2008 era associates with compromised outcomes for DCD procedures in comparison to DBD procedures (i.e. higher incidences of DGF and early graft loss, impaired 1-year renal function in patients without DGF, and inferior 1- and 5-year graft survival rates), the 2008-2018 era shows outcome equivalence for DBD and DCD procedures. Only the incidence of DGF for DCD grafts remained high in the current era (Table 2), but this did not impact graft and patient survival. Yet, for both timeframes and for both donor types, DGF resulted in a 15% reduced 1-year renal function (Table 2).

To explore whether the shift towards outcome equivalence reflects effect modification by time, an interaction (Wald) test was performed. As expected, the interaction test confirmed a significant difference in the effect of donor type on outcomes (DGF, early graft loss, 1- and 5-year graft survival) between the two time eras (p for interaction: <0.001, 0.002, 0.001 and <0.001, respectively) (Table 2).

Table 1. Baseline characteristics of deceased-donor kidney transplants in The Netherlands for the 1998-2008 and the 2008-2018 interval.

		01/1998 - 12/2007			01/2008 - 12/2017			
		DBD n = 2361 (67.5%)	**DCD** n = 1138 (32.5%)	**p-value**	**DBD** n =1929 (51.0%)	**DCD** n = 1852 (49.0%)	**p-value**	**p for interaction**
BASELINE	**Donor age**	46.2 ± 15.3	45.4 ± 15.4	*0.16*	52.7 ± 14.6	51.6 ± 14.5	*0.03*	***<0.001***
	Recipient age	48.6 ± 14.3	50.7 ± 13.3	*<0.001*	55.0 ± 14.2	55.9 ± 12.9	*0.03*	***<0.001***
	Time on dialysis (years)	4.1 ± 2.5	4.2 ± 2.0	*0.24*	3.8 ± 2.5	3.6 ± 2.1	*0.001*	***<0.001***
	First warm ischaemic period (min.)	NA	19.0 [15.0 - 25.0]	-	NA	16.0 [13.0 - 19.0]	-	-
	Cold ischaemic period (hours)	20.0 [15.6 - 24.6]	20.0 [16.0 - 23.8]	*0.32*	15.4 [11.8 - 19.8]	14.0 [11.5 - 17.6]	*<0.001*	***<0.001***
	≤24 hours	1705 (74.3%)	889 (79.9%)		1589 (92.2%)	1575 (96.8%)		
	>24 hours	589 (25.7%)	224 (20.1%)		135 (7.8%)	52 (3.2%)		
	Graft anastomosis time (min.)	34.0 [27.0 - 41.0]	33.0 [27.0 - 40.0]	*0.32*	33.0 [26.0 - 41.0]	32.0 [25.0 - 40.0]	*0.01*	***0.01***
	KDRI [1]	1.23 [1.0 - 1.5]	1.35 [1.1 - 1.6]	*<0.001*	1.39 [1.1 - 1.7]	1.49 [1.2 - 1.8]	*<0.001*	***<0.001***
	Preservation solution			*<0.001*			*<0.001*	***<0.001***
	- HTK	325 (13.9%)	967 (86.1%)		463 (24.4%)	719 (39.3%)		
	- University of Wisconsin	2001 (85.4%)	152 (13.5%)		1383 (72.8%)	1058 (57.8%)		
	- Other	17 (0.7%)	4 (0.4%)		55 (2.9%)	53 (2.9%)		

Data are respectively presented as mean ± standard deviation, as number (%), as median [interquartile range] or as beta coefficient (β), odds ratio or hazard ratio with the corresponding 95% confidence interval.

[1] Multiple imputation was applied for missing data of variables included in the KDRI.

DBD, donation after brain death; DCD, donation after circulatory death; HTK, histidine-tryptophan-ketoglutarate; KDRI, kidney donor risk index; min., minutes; NA, not applicable.

Table 2. Deceased-donor kidney transplant outcomes in The Netherlands for the 1998-2008 and the 2008-2018 interval.

		01/1998–12/2007			01/2008–12/2017			
		DBD n = 2361 (67.5%)	**DCD** n = 1138 (32.5%)	**p-value**	**DBD** n =1929 (51.0%)	**DCD** n = 1852 (49.0%)	**p-value**	**p for interaction**
OUTCOME	**Delayed graft function**	**420 (17.8%)**	**511 (44.9%)**	***<0.001***	**321 (16.6%)**	**736 (39.7%)**	***<0.001***	
	Crude OR (95% CI)	Ref.	4.04 (3.42–4.78)	*<0.001*	Ref.	3.85 (3.28–4.53)	*<0.001*	***<0.001***
	Adjusted OR (95% CI)	Ref.	4.17 (3.45 – 5.04)	*<0.001*	Ref.	4.78 (3.99 – 5.70)	*<0.001*	
	Early graft loss (<day 90)	**194 (8.2%)**	**151 (13.3%)**	***<0.001***	**110 (5.7%)**	**114 (6.2%)**	***0.56***	
	Crude OR (95% CI)	Ref.	1.71 (1.36–2.14)	*<0.001*	Ref.	1.09 (0.83–1.42)	*0.56*	***0.002***
	Adjusted OR (95% CI)	Ref.	1.77 (1.40 – 2.23)	*<0.001*	Ref.	1.24 (0.92–1.68)	*0.16*	
	- Primary non-function	44 (22.7%)	58 (38.4%)		42 (38.2%)	43 (37.7%)		
	- Rejection	58 (29.9%)	25 (16.6%)		23 (20.9%)	20 (17.5%)		
	- Thrombosis or infarction	38 (19.6%)	36 (23.8%)		14 (12.7)	24 (21.1%)		
	- Other	54 (27.8%)	32 (21.2%)		31 (28.2%)	27 (23.7%)		
	1-year eGFR DGF -	**52.6 ± 19.7**	**49.5 ± 18.1**	***0.02***	**51.5 ± 19.7**	**52.3 ± 20.0**	***0.44***	
	Crude β (95% CI)	Ref.	-3.07 (-5.67–-0.47)	*0.02*	Ref.	0.84 (-1.29–2.96)	*0.44*	***0.93***
	Adjusted β (95% CI)	Ref.	-4.21 (-6.57–-1.86)	*<0.001*	Ref.	-0.11 (-2.04 – 1.82)	*0.91*	
	1-year eGFR DGF +	**44.1 ± 19.0**	**44.4 ± 18.4**	***0.81***	**44.8 ± 18.9**	**44.6 ± 17.5**	***0.89***	
	Crude β (95% CI)	Ref.	0.32 (-2.19–2.82)	*0.81*	Ref.	-0.18 (-2.81–2.45)	*0.89*	***0.70***
	Adjusted β (95% CI)	Ref.	-0.69 (-3.02 – 1.63)	*0.56*	Ref.	-1.63 (-4.05 – 0.79)	*0.19*	
	1-year graft loss	(10.9%)	(15.0%)		(8.0%)	(7.9%)		
	Crude HR (95% CI)	**1.0**	**1.41 (1.16–1.71)**	***0.001***	**1.0**	**0.98 (0.78–1.23)**	***0.84***	***0.001***
	Adjusted H R (95% CI)	1.0	1.45 (1.19–1.77)	*<0.001*	1.0	1.09 (0.85–1.40)	*0.49*	
	5-year graft loss	(20.0%)	(22.8%)		(14.0%)	(13.6%)		
	Crude HR (95% CI)	**1.0**	**1.18 (1.01–1.37)**	***0.04***	**1.0**	**0.96 (0.80–1.15)**	***0.66***	***<0.001***
	Adjusted HR (95% CI)	1.0	1.25 (1.07 – 1.46)	*0.01*	1.0	1.04 (0.85–1.27)	*0.69*	
	1-year patient survival	(95.0%)	(94.4%)		(95.2%)	(94.8%)		
	Crude HR (95% CI)	**1.0**	**1.14 (0.84–1.54)**	***0.41***	**1.0**	**1.08 (0.82–1.43)**	***0.58***	***0.60***
	Adjusted HR (95% CI)	1.0	1.08 (0.80–1.46)	*0.62*	1.0	1.08 (0.81–1.42)	*0.61*	
	5-year patient survival	(82.8%)	(82.4%)		(82.3%)	(82.5%)		
	Crude HR (95% CI)	**1.0**	**1.04 (0.88–1.23)**	***0.66***	**1.0**	**1.00 (0.85–1.17)**	***0.96***	***0.81***
	Adjusted HR (95% CI)	1.0	1.08 (0.89–1.30)	*0.44*	1.0	1.02 (0.85–1.22)	*0.83*	

Data are respectively presented as mean ± standard deviation, or as number (%), and beta coefficient (β), odds ratio or hazard ratio with the corresponding 95% confidence interval.

95% CI, 95% confidence interval; DBD, donation after brain death; DCD, donation after circulatory death; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; HR, hazard ratio; min., minutes; OR, odds ratio.

Discussion

This national study demonstrates a major improvement in outcomes of transplanted DCD kidneys over time. Whereas the 1998-2008 era associates with inferior outcomes for DCD kidney transplant procedures, the 2008-2018 era shows outcome equivalence between DBD and DCD kidney transplants. This shift underpins that DCD kidney transplantation has come of age and results are nowadays comparable to DBD kidney transplants. It also emphasises that conclusions based on retrospective data (i.e. based on timeframes in which outcomes of DCD procedures were inferior to DBD procedures) are interfered by time-varying confounding and effect modification, and are therefore no longer justified.

Patient- and graft survival equivalence for DBD and DCD procedures occurred despite a persistent high incidence of DGF in DCD grafts. This apparent paradox can be explained by differential impacts of DGF on DBD and DCD outcomes, with a negligible impact of DGF on patient- and graft survival in recipients with DCD grafts.[4,14] A phenomenon that presumably relates to donor type-specific molecular differences in organ resilience.[14]

A clear, univocal explanation for the improved outcomes is missing. In the context of more adverse donor and recipient risk profiles in the more recent timeframe, the improvement in DCD outcomes over time presumably involves a complex interplay of factors that includes optimized surgical procedures and immunosuppressive regimens, altered organ preservation techniques, and enhanced transport logistics.[15-19] Certainly, a significant impact has been the profound reduction in cold ischemic time.[16,20] Several studies have shown that a prolonged cold ischemic time is more deleterious in recipients receiving kidney transplants from DCD donors than in recipients from DBD donors.[21-23] This finding, with DCD grafts being more 'vulnerable' to cold ischemia than DBD grafts, has recently been confirmed by colleagues in The Netherlands,[20] and might also explain why graft survival rates have improved to a greater extent for recipients of DCD donors than for recipients of DBD donors. Another possible explanation is that–as cold ischemic periods of more than 24 hours in DCD kidneys are associated with worse graft survival–the proportion of procedures with excessive cold ischemic periods (>24 hours) decreased over time with the increasing awareness of avoiding long cold ischemic times in The Netherlands.[4] A potential contribution of hypothermic machine perfusion (HMP) to improved outcomes, however, is limited in this Dutch cohort since HMP was only fully implemented in the year 2016, and as the available data indicate that although HMP reduces the risk of DGF, it has a limited impact on the other outcome data.[24]

Improved outcomes over time may further reflect advances in immunosuppressive therapies including the conversion from cyclosporine to tacrolimus as standard

maintenance regime.[25,26] Also, the introduction of more sensitive techniques to detect anti-human leukocyte antigen antibodies, such as the LUMINEX technique may have resulted in increased graft survival.[18]

An alternative and non-exclusive explanation for the improved outcomes is the presence of a learning curve that involves the intangible and often intuitive aspects of medical decision-making processes in the context of donor and recipient selection, and organ allocation. Existence of a learning curve phenomenon is supported by the observation that outcomes of transplanted DBD kidneys improved significantly in a similar way as for DCD transplant procedures, but at an earlier (1990-1998) time era, and by the dynamics of the incidence of early graft loss over time (Figure 1). To be more specific, data for DBD procedures show a steep decline and stabilization of the incidence of early graft loss after 1998. A similar–albeit postponed–pattern is seen for the DCD procedures, for which the early graft loss incidence rate dropped and stabilized following 2008. Thus, it is likely that countries initiated a controlled DCD programme, may also experience some form of learning curve with transient inferior outcomes for this type of transplant procedure.

Remarkably, the time-related improvements in DCD outcomes occurred despite considerable increases in donor and recipient age, and in KDRI over time (Table 1). The apparent paradox of increasing KDRI values but improving graft survival rates suggests that, after the medical decision to accept a kidney for donation, there is a limited impact of donor characteristics on graft survival. This is illustrated by the remarkably low Nagelkerke R^2 for the association between KDRI and 5-year graft survival.[13] Hence, these data illustrate that graft survival reflects an interplay of donor, procedural and recipient factors.[15]

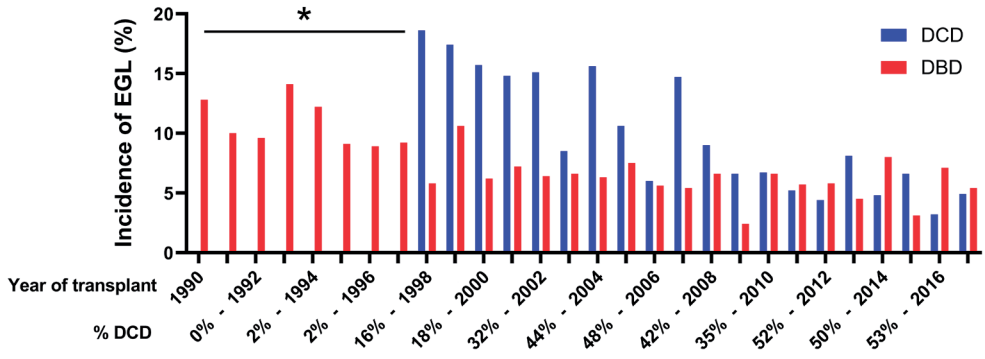

Figure 1. Time-related incidences of early graft loss in 8,511 primary kidney transplant recipients according to deceased donor type in The Netherlands. * The small number of DCD kidney transplant procedures performed in these years (n < 15), does not justify adequate power for analysis. *DBD, donation after brain death; DCD, donation after circulatory death; EGL, early graft loss.*

This study has some limitations. Firstly, it is a registry-based study, which is associated with inherent design limitations. Secondly, this is a country-specific study as outcomes are influenced by national guidelines and decision-making policies. However, considering the liberal attitude towards DCD kidneys in The Netherlands (reflected by an equal distribution in DBD and DCD procedures, comparable donor ages and KDRI values) it is unlikely that the results reflect a high threshold in accepting DCD grafts.

In conclusion, this registry-based study shows a major improvement in outcomes of transplanted DCD kidneys over time, with DBD and DCD outcome equivalence in the current timeframe. The time-related improvements for DCD outcomes not only show that DCD kidneys can be fully embraced, but also emphasise that careful interpretation is required for conclusions that are based on historical cohorts.

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Supplemental Table 1. Multivariate analyses of posttransplant outcomes.

	Donor age	Recipient age	Time on dialysis	Cold ischemic period	Graft anastomosis time
Delayed graft function					
1998-2008 (OR + 95% CI)	1.010 (1.004–1.017)**	1.002 (0.995–1.008)	1.069 (1.029–1.110)**	-	1.007 (1.001–1.014)*
2008-2018 (OR + 95% CI)	1.013 (1.007–1.020)**	-	1.106 (1.065–1.148)**	1.030 (1.015–1.046)**	-
Early graft loss (<day 90)					
1998-2008 (OR + 95% CI)	1.018 (1.010–1.027)**	-	-	1.032 (1.015–1.049)**	1.017 (1.009–1.024)**
2008-2018 (OR + 95% CI)	1.028 (1.014–1.041)**	1.002 (0.990–1.015)	1.100 (1.037–1.167)**	1.027 (1.001–1.053)*	1.018 (1.008–1.028)**
1-year eGFR DGF -					
1998-2008 (β + 95% CI)	-0.535 (-0.593–-0.476)**	-0.153 (-0.217–-0.088)**	-	-0.308 (-0.439–-0.176)**	-
2008-2018 (β + 95% CI)	-0.512 (-0.577–-0.448)**	-0.112 (-0.181–-0.042)**	-	-	-
1-year eGFR DGF +					
1998-2008 (β + 95% CI)	-0.488 (-0.566–-0.409)**	-0.061 (-0.145–0.022)	-	-	-
2008-2018 (β + 95% CI)	-0.527 (-0.611–-0.442)**	-0.006 (-0.093–0.080)	-	-	-
1-year graft loss					
1998-2008 (HR + 95% CI)	1.021 (1.014–1.028)**	-	-	1.029 (1.016–1.043)**	1.012 (1.007–1.018)**
2008-2018 (HR + 95% CI)	1.026 (1.015–1.037)**	1.001 (0.991–1.011)	1.080 (1.028–1.134)**	1.023 (1.002–1.045)*	1.012 (1.004–1.021)**
5-year graft loss					
1998-2008 (HR + 95% CI)	1.024 (1.018–1.030)**	0.986 (0.981–0.992)**	-	1.022 (1.011–1.033)**	1.008 (1.003–1.012)**
2008-2018 (HR + 95% CI)	1.018 (1.011–1.026)**	-	1.043 (1.001–1.087)*	1.024 (1.007–1.041)*	1.013 (1.007–1.020)**
1-year patient survival					
1998-2008 (HR + 95% CI)	1.015 (1.005–1.026)**	1.049 (1.035–1.063)**	-	-	-
2008-2018 (HR + 95% CI)	1.006 (0.995–1.017)	1.049 (1.034–1.064)**	-	-	-
5-year patient survival					
1998-2008 (HR + 95% CI)	1.018 (1.011–1.024)**	1.049 (1.040–1.058)**	1.071 (1.033–1.112)**	0.992 (0.978–1.006)	1.003 (0.997–0.010)
2008-2018 (HR + 95% CI)	1.011 (1.003–1.018)**	1.057 (1.047–1.067)**	1.083 (1.044–1.123)**	1.021 (1.005–1.036)*	1.012 (1.005–1.018)**

In total 16 multivariate analyses were performed. Each row represents a single multivariate analysis. The multivariate models were adjusted for variables that were statistically relevant in the univariate analysis (p-value <0.1).

(-) Indicates that the variable was not included in the multivariate analysis. (*) p-value <0.05, (**) p-value <0.005.

95% CI, 95% confidence interval; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; HR, hazard ratio; OR, odds ratio.

Chapter 4

The neglectable impact of delayed graft function on long-term graft survival in kidneys donated after circulatory death associates with superior organ resilience

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Abstract

Objective: To explore putative different impacts of delayed graft function (DGF) on long-term graft survival in kidneys donated after brain death (DBD) and circulatory death (DCD).

Background: Despite a 3-fold higher incidence of DGF in DCD grafts, large studies show equivalent long-term graft survival for DBD and DCD grafts. This observation implies a differential impact of DGF on DBD and DCD graft survival. The contrasting impact is remarkable and yet unexplained.

Methods: The impact of DGF on DBD and DCD graft survival was evaluated in 6635 kidney transplants performed in The Netherlands. DGF severity and functional recovery dynamics were assessed for 599 kidney transplants performed at the Leiden Transplant Center. Immunohistochemical staining, gene expression profiling, and Ingenuity Pathway Analysis were used to identify differentially activated pathways in DBD and DCD grafts.

Results: While DGF severely impacted 10-year graft survival in DBD grafts (HR 1.67; $P < 0.001$), DGF did not impact graft survival in DCD grafts (HR 1.08; $P = 0.63$). Shorter dialysis periods and superior posttransplant eGFRs in DBD grafts show that the differential impact was not caused by a more severe DGF phenotype in DBD grafts. Immunohistochemical evaluation indicates that pathways associated with tissue resilience are present in kidney grafts. Molecular evaluation showed selective activation of resilience-associated pathways in DCD grafts.

Conclusions: This study shows an absent impact of DGF on long-term graft survival in DCD kidneys. Molecular evaluation suggests that the differential impact of DGF between DBD and DCD grafts relates to donor-type specific activation of resilience pathways in DCD grafts.

Introduction

In an era of severe donor organ shortage and growing waiting lists for renal transplantation there is an increased reliance on expanded criteria donors and organs donated after circulatory death (DCD). While DCD donor kidneys constitute a large potential donor pool, higher incidences of primary non function and particularly delayed graft function (DGF) are regarded as major impediments.

Notwithstanding the higher incidence of DGF in DCD compared to donated after brain death (DBD) grafts, large cohort studies from the United Kingdom and The Netherlands show equivalent survival for kidneys DBD and DCD grafts.[1-3] This observation suggests a differential impact of DGF on DBD and DCD graft survival.

The apparent differential impact of DGF on DBD and DCD graft survival is remarkable and yet unexplained. One possible explanation for this phenomenon is that the type of DGF in DBD grafts reflects more severe transplantation-related injury. An alternative and mutually nonexclusive explanation is that the differential impact reflects differences in graft "resilience"—i.e. the ability of the graft to cope with negative environmental changes[4]—with DCD donor kidneys being more "resilient" than DBD grafts. Tissue resilience is an established phenomenon in cancer biology, and negatively associates with patient prognosis.[4] However, in the context of transplantation biology, resilience could be a beneficial factor potentially contributing to better transplantation outcomes.

Considering the emerging epidemiological evidence for a different impact of DGF on DBD and DCD graft survival and its clinical relevance, we have focused in this hypothesis generating study on this putative differential impact and also attempted to explore its biological basis.

Materials and methods

Study population

The impact of DGF (defined as the need for dialysis in the first postoperative week(s)) on long-term graft survival was evaluated in 6635 deceased donor kidney transplants performed between January 2000 and January 2018 in the Netherlands (Netherlands Organ Transplant Registry (NOTR)). Combined organ procedures, procedures in recipients younger than 12 years and uncontrolled circulatory death donor procedures were excluded.

The impact of donor type on DGF phenotype and functional recovery dynamics was assessed for 287 DBD and 312 DCD kidney transplants performed at the Leiden University Transplant Center between 2007 and 2018. A more detailed description of the methods is given in the Supplemental Data.

The clinical nomenclature and different phases included in this paper are illustrated in Figure 1.

Histology and gene expression

Pre-reperfusion tissue biopsy samples from 80 donor kidneys were randomly selected based on donor type and the presence or absence of DGF (n = 20 per group, Supplemental Table 1). Immunohistochemical staining was performed for BCL2, IGF-1R, p53, PCNA, phospho-EGFR, phospho-MAPK14, phospho-mTOR, PPARγ. Details of the antibodies and procedures are summarized in the Supplemental Data and Supplemental Table 2.
Gene expression profiling of 23 DBD and 16 DCD pre-reperfusion renal biopsies was followed by Ingenuity Pathway Analysis (IPA, QIAGEN, USA) to identify differentially regulated pathways (Supplemental Table 3).[5,6]
All renal biopsies used in this study were collected after static cold storage, and prior to reperfusion. Further details of the analyses are provided in the Supplemental Data.

Statistical analysis

STATA/SE version 12.0 (StataCorp, Texas) and IBM SPSS Statistics 23.0 (Amsterdam, The Netherlands) were used for statistical analysis. Comparisons between groups were analyzed using standard statistical methods. Cox proportional hazards models, adjusted for donor/recipient age and sex, and cold ischemic period were used to evaluate differences in impact of DGF on 10-year graft survival. Univariate analysis was followed by multivariate regression analysis to identify factors associated with DGF. A detailed description of the statistical analysis is given in the Supplemental Data.

Results

Epidemiological evaluation

Putative differential impacts of DGF on DBD and DCD graft survival were evaluated in 6635 kidney transplants (43.6% DCD procedures) that were performed between 2000 and 2018 in The Netherlands (Supplemental Table 4). The registry data confirmed a higher incidence of DGF in DCD grafts (DCD: 42.2% vs. DBD: 17.8%; $P < 0.001$) but also showed differential impact of DGF on long-term graft survival per donor type. In fact, while DGF severely impacted 10-year graft survival in DBD donor kidneys [adjusted DGF-associated hazard ratio (aHR) for graft loss: 1.67 (95% CI 1.35–2.08); $P < 0.001$], no impact on survival was observed for DGF in DCD donor kidneys [aHR for graft loss: 1.08 (95% CI 0.82–1.39); $P = 0.63$]. Interaction testing confirmed the differential impact of DGF on DBD and DCD long-term graft survival (P for interaction < 0.001).

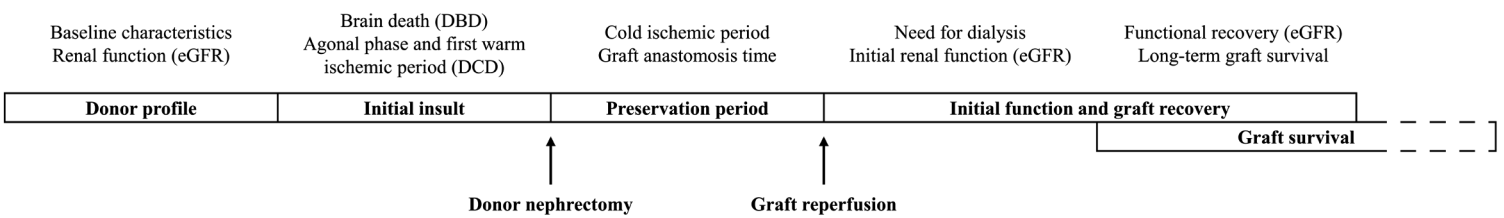

Figure 1. The clinical nomenclature and different phases included in this paper.

The differential impact of DGF on long-term graft survival may relate to a greater threshold to develop DGF in DBD grafts (i.e., that development of DGF in DBD grafts requires a more severe

insult). This hypothesis was tested by using a qualitative and quantitative evaluation of risk factors associated with DGF. An inventory of risk factors associated with occurrence of DGF (multivariate analyses) revealed clearly qualitative differences between the 2 donor types. The first warm ischemic period, a discriminant factor of DCD grafts, was positively associated with DGF in DCD grafts. Both donor types shared cold ischemic period as a risk factor for developing DGF. Donor age was a significant risk factor for DBD grafts, but an association with DGF in DCD grafts did not reach statistical significance ($P = 0.11$). The last serum creatinine value in the donor, human leukocyte antigen (HLA)-DR mismatch, and graft anastomosis time exclusively associated with DGF in DBD grafts but not in DCD grafts (Supplemental Table 5).

Quantitative analysis showed that DGF in recipients of DBD grafts was associated with a slightly unfavorable donor and procedural profile as reflected by the 2-year difference in donor age, higher donor serum creatinine concentrations, and 8% and 12% longer cold ischemic and graft anastomosis times (Table 1). However, this less favorable risk profile did not result in a more severe DGF phenotype in DBD grafts. On the contrary, recipients of DCD grafts with DGF required longer dialysis, and had profoundly inferior renal function (eGFR) in the first week following the last dialysis ($P < 0.001$) (Table 2).

The above results did not point to a more profound DGF phenotype as underlying cause of the negative impact of DGF on long-term graft survival in DBD grafts. Alternatively, the differing impact may reflect differential resilience between the 2 donor types, with DCD grafts being more resilient than DBD grafts. A concept that is supported by the superior functional (eGFR) recovery dynamics in DCD grafts (Figure 2).

Histology and gene expression

To explore the presence of resilient enhancing factors, we mapped several molecular upstream regulators associated with resilience in the context of tumor biology (e.g., p53, phospho-EGFR, IGF-1R, phospho-mTOR, phospho-MAPK14, PCNA, BCL2 and PPARγ).[7–13] The immunohistochemical analysis demonstrated expression of the aforementioned resilience factors in pre-reperfusion kidney biopsies, indicating that aspects of the molecular mechanisms associated with tissue resilience are present in both donor types (Supplemental Figures 1 and 2).

With the aim of evaluating putative differential activation of molecular pathways associated with resilience in DBD and DCD grafts, an unbiased pathway analysis was

Table 1. Comparison of risk factors associated with DGF in DBD and DCD graft recipients.

	DBD DGF + n = 667	DCD DGF + n = 1219	p-value
Donor age (years)	52.1 (14.4)	50.2 (14.5)	0.006
Donor last creatinine (µmol/L)	77.0 [60.0–100.0]	68.0 [54.0–83.5]	< 0.001
Mismatch			0.004
HLA-DR 0	243 (36.5%)	362 (29.9%)	
1	360 (54.1%)	752 (62.0%)	
2	62 (9.3%)	98 (8.1%)	
Cold ischemic period (hours)	18.4 [14.4–23.0]	17.0 [13.1–21.0]	< 0.001
Graft anastomosis time (min.)	35.0 [26.0 – 42.0]	31.0 [25.0 – 40.0]	< 0.001

Data are presented as mean ± standard deviation (SD) or as number (%) or as median [25 and 75 IQR].

Table 2. DGF phenotype in DBD and DCD graft recipients.

	DBD DGF + n = 80	DCD DGF + n = 179	p-value
Duration of dialysis (days)	7.5 [5.0–12.0]	9.0 [6.0–13.8]	0.039
Number of dialysis	3.5 [3.0–5.8]	4.0 [3.0–6.0]	0.462
First autonomous eGFR	20.3 [14.4–35.7]	13.4 [9.3–22.8]	< 0.001

Data are presented as median [25 and 75 IQR].

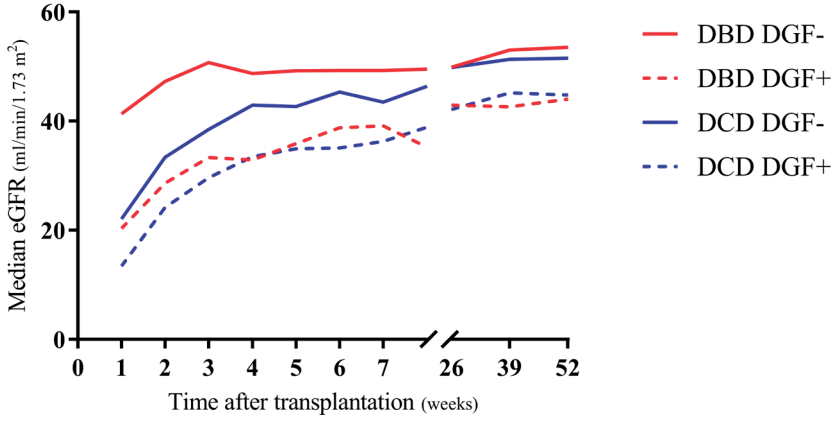

Figure 2. Functional renal recovery (eGFR) after kidney transplantation.

performed on the gene expression profiles in pre-reperfusion kidney biopsies from DBD and DCD donors. There were no differences in baseline characteristics between DBD and DCD donors (Supplemental Table 3, http://links.lww.com/SLA/B725). Using DBD grafts as the comparator, 6 differentially activated (P < 0.05) upstream regulatory

pathways, and 13 differentially inhibited regulatory pathways were identified in DCD grafts (Figure 3). All upregulated pathways belonged to a family of factors responsible for renal development, cell fate, organogenesis, and stem cell maintenance. Pathways inhibited in DCD grafts included the p53 pathway, and a cluster of pro-inflammatory factors (IL6, TNFα, RANKL (TNFSF11), CEBPβ, TICAM1) (Figure 3). Functionally, the strongest influence was found by pathways associated with cardio-vascular diseases (P value range 2.5 x 10^{-10} to 2.2 x 10^{-3}), in particular a gene cluster mapped by IPA as "advanced stage peripheral artery disease" (P value 2.5 x 10^{-10}). This cluster is dominated by upregulation of heat shock proteins (Supplemental Figure 3).

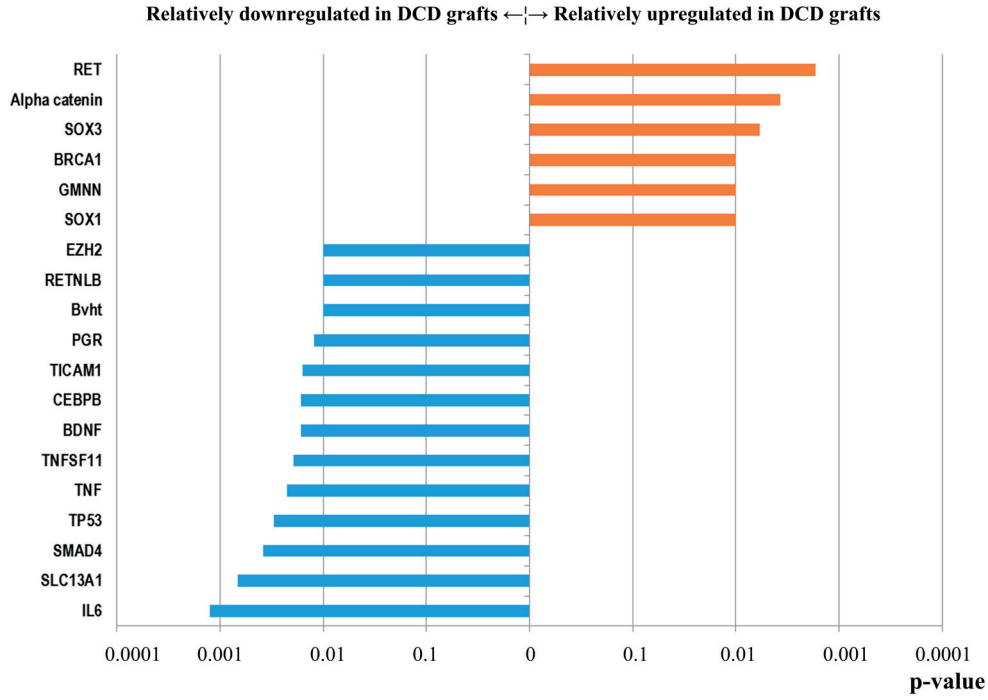

Figure 3. Differentially regulated upstream regulators in DBD and DCD donor kidneys based on Ingenuity Pathway Analysis (DBD is reference).
BDNF (Brain-derived neurotrophic factor); BRCA1 (Breast cancer-associated gene 1); Bvht (Braveheart); CEBPB (CCAAT/Enhancer Binding Protein-β); EZH2 (Enhancer of zeste homolog 2); GMNN (Geminin); IL6 (Interleukin-6); PGR (Progesteron Receptor); RET (Rearranged during transfection); RETNLB (Resistin-like molecule β); SLC13A1 (Solute carrier family 13 member 1); SMAD4 (Sma (*Caenorhabditis elegans*) Mothers Against Decapentaplegia homologue 4); SOX1 (Sex determining region Y-box protein 1); SOX3 (Sex determining region Y-box protein 3); TICAM1 (Toll Like Receptor Adaptor Molecule 1); TNFα (Tumor Necrosis Factor α); TNFSF11 (Tumor Necrosis Factor ligand Superfamily member 11); TP53 (Tumor Protein p53).

Discussion

Whilst a high incidence of DGF after DCD kidney transplantation is considered a major obstacle toward a more liberal use of these grafts, recent epidemiological observations suggest that this concern might be unjustified. This integrative epidemiological and molecular analysis has clearly shown a differential impact of DGF on DBD and DCD graft survival, with no impact of DGF on DCD graft survival. This finding may reflect a more favorable baseline molecular resilience signature in DCD donor kidneys.

Transplants procedures with DCD donor kidneys are associated with a twofold to threefold increased incidence of DGF.[2,3,14] DGF is an established risk factor for premature graft loss, and as such the higher incidence of DGF with DCD grafts is considered a relative contra-indication for the use of DCD grafts by some transplant centers. This notion has recently been challenged by cohort studies showing equivalent graft survival for DBD and DCD grafts despite the difference in incidence of DGF: an observation that implies a differential impact of DGF on DBD and DCD graft survival. In this context it should be noted that the conclusions regarding the negative association between DGF and long-term outcomes are mainly based on studies from an era with an almost exclusive use of DBD grafts.[15–19] Moreover, it cannot be excluded that conclusions for DCD grafts are confounded by factors that relate to both DGF and graft survival.

The differential impact of DGF on graft survival was confirmed by the outcome data for almost 6,700 deceased donor kidney transplantations performed in The Netherlands, a country with a longstanding liberal tradition toward the use of DCD grafts (currently 50% of all deceased kidney transplantation procedures). While regression analysis confirmed the impact of DGF on long-term graft survival in DBD grafts, DGF did not affect graft survival in DCD grafts.

In an effort to understand the different impact of DGF on graft survival we first tested in this study whether the apparent impact on DBD grafts reflects the presence of a more severe DGF phenotype. This hypothesis was not supported by the clinical data. On the contrary, transplants with DCD grafts were hallmarked by a more severe graft injury as indicated by profoundly impaired posttransplant renal function (eGFR), and in case of DGF, a prolonged need for posttransplant dialysis. Irrespective of this, DCD grafts demonstrated an adequate functional recovery within weeks after transplantation, resulting in a renal function fully comparable to DBD grafts. The impact of DGF on ultimate eGFR was similar for DBD and DCD grafts. Thus, our clinical data do not support a more severe DGF phenotype as underlying cause of the negative impact of DGF in DBD grafts. In this light, we explored possible differences in graft resilience as an alternative explanation for the contrasting impact of DGF in DBD and DCD grafts.

Biologically, resilience is the ability of an organism to recover to normal functioning after perturbation.[20] In the context of ageing, resilience is the ability to cope with stress and re-establish homeostasis.[21] Tissue resilience is an established phenomenon in tumor biology, and a known negative prognostic factor.[4] In the context of organ transplantation, superior resilience would obviously be beneficial in terms of graft recovery and survival.

We applied gene expression profiling followed by pathway analysis to map putative molecular differences in organ resilience between DBD and DCD grafts. Pathways relatively enriched (n = 6) in DCD grafts were all part of established resilience networks. Five upregulated pathways in DCD grafts (RET, Alpha catenin, GMNN, SOX1, and SOX3) were associated with renal development and cell proliferation, and partly associate with the Wnt/β -catenin signalling pathways:[22–26] a pivotal pathway in kidney development, repair, and regeneration.[27–32] The sixth upregulated pathway was the BRCA1 tumor suppressor pathway. BRCA1 is a key player in cellular repair through its role in DNA repair and cell cycle checkpoint activation. This pathway was recently shown to be cardioprotective after myocardial infarction.[33] In contrast to the BRCA-1 tumor suppressor pathway, we observed down-regulation of the p53 network. While this downregulation is considered a negative aspect in tumor biology, it has been pointed out that downregulation of p53 is part of the normal, physiological regenerative response, and as such, could be part of an activated resilience network.[34]

Downregulated pathways in DCD grafts were dominated by pro-inflammatory signaling cascades (i.e., IL6, TNFα, RANKL (TNFSF11), CEBPβ, TICAM1). This downregulation could be a consequence of an activated resilience network in DCD grafts. Other explanations included passive enrichment, reflecting differences in leucocyte influx (and thus genes associated with leucocytes) in DBD grafts,[35] as well as upregulation of parenchymal inflammation in response to brain death in DBD grafts.[36] It is unclear to what extent the relative downregulation of inflammatory responses in DCD grafts contributes to the absent impact of DGF in these grafts. Although inflammation is often seen as a "negative" factor, experimental data suggests that brain death-associated immune activation may not accelerate ischemia reperfusion injury[37] whereas other studies actually indicate aggravation of experimental ischemia reperfusion injury following interference with IL-6 or IL-9 signaling.[35,38]

A further observation is the downregulation of the BDNF signalling route in DCD grafts. Strong associations exist between BDNF and the kidney injury molecule (KIM-1), and BDNF has been recently proposed as a biomarker for glomerular injury.[39] As such, the relative downregulation of BDNF in DCD grafts might indicate that the glomerular injury is less in DCD than in DBD grafts.

On the functional level, the most influential transcriptomic signals were related to cardiovascular diseases, in particular "advanced stage peripheral artery disease." This cluster is mainly comprised of members of heat shock protein superfamily. Induction of heat shock proteins following ischemia has been well documented. In the context of brain ischemia this was correlated with the regions that ultimately survived the injury,[40] suggesting that this superfamily is part of a resilience response.

Since all renal biopsies in this study were from grafts that were maintained on static cold storage (hence a state of absent transcriptional activity), the clear differences in gene expression profiles probably reflect donor-specific aspects such as brain death.[41] An alternative and nonexclusive explanation is that the activation of resilience pathways in DCD grafts is caused by a process of ischemic preconditioning that may occur during the agonal phase and first warm ischemic period prior to donor nephrectomy in DCD donors. Ischemic preconditioning, which generally refers to a preceding state of ischemia that is followed by reperfusion, is an established phenomenon in experimental studies.[42–44] Yet, studies so far do not indicate a benefit of ischemic preconditioning for clinical kidney injury.[45] It might be speculated that the ischemia applied in clinical studies is insufficient to induce activation of resilience pathways, and that more profound and localized triggers which occur during the agonal phase and first warm ischemic period in DCD donors are required.

Our study has several limitations. It is in part based on registry data including the standard flaws of a registry with some data missing and a lack of predefined variables, leading to more heterogeneity in data registration. Outcomes are prone to confounding by indication with some clinicians being more critical than others when accepting or declining DCD grafts for transplantation. Also, exploration of molecular mechanisms is based on observational data. A more detailed experimental exploration and validation of the observed differences is compromised by the profound species differences with regard to acute injury, ischemia reperfusion, and resilience.[46,47]

In conclusion, results in this clinically relevant study show that DGF has no obvious impact on long-term graft survival in DCD grafts. As such, the high incidence of DGF in DCD grafts should not be regarded a relative contraindication or impediment toward the use of these donor kidneys. The molecular evaluation performed suggests that the different impact of DGF in DBD and DCD grafts relates to donor type-specific regulation of resilience and pro-inflammatory pathways benefitting the DCD graft and its outcomes.

Supplemental data

Materials and methods

Study population

The impact of DGF on 10-year graft survival was evaluated in 6,635 deceased-donor kidney transplantations performed between 2000 and 2018 (Netherlands Organ Transplant Registry (NOTR)). Combined organ procedures, procedures in recipients younger than 12 years and uncontrolled circulatory death donor procedures were excluded.

The impact of DBD and DCD donor type on DGF phenotype and functional recovery dynamics was assessed for 287 DBD and 312 DCD kidney transplantations performed at the Leiden University Transplant Center between 2007 and 2018. In addition to previously described exclusion criteria, we excluded grafts with primary non-function. DGF was defined as the need for dialysis in the first postoperative week(s), followed by functional recovery and with exception of a single dialysis performed for elevated potassium levels or fluid overload. The MDRD (Modification of Diet in Renal Disease) Study equation was used to estimate glomerular filtration rate (eGFR). For recipients with DGF, the first autonomous eGFR ('week 1') was defined as the first week following last dialysis. Factors included in the DGF phenotype were duration of dialysis, number of dialysis and the first autonomous eGFR.

Histology and gene expression

Routine pre-reperfusion graft biopsies are used for graft quality control. All biopsies were collected after static cold storage, and prior to reperfusion. Biopsy samples from 80 donor kidneys were randomly selected based on donor type and the presence or absence of DGF (n=20 per group, Supplemental Table 1). One biopsy contained non-renal tissue and was excluded. Tissue sections (4μm) were cut and immunohistochemical staining was performed for BCL2, IGF-1R, p53, PCNA, phospho-EGFR, phospho-MAPK14, phospho-mTOR, PPARγ. Details of the antibodies and procedures are summarized in Supplemental Table 2. Anti-rabbit/mouse EnVision+ (Dako, Amstelveen, The Netherlands) and 3,3'-diaminobenzidine (DAB) substrate chromogen system (Dako, Amstelveen, The Netherlands) were used for antibody visualization. Tissue sections were counterstained with hematoxylin (Klinipath, Duiven, The Netherlands). All tissue sections were semi-quantitatively and independently reviewed by two observers (M.K. and J.T.N) and scored as: 0 (none), 1 (minimal), 2 (slight), 3 (moderate) and 4 (abundant). Scoring disagreements were identified and resolved by joint review to achieve consensus.

Gene expression profiling of pre-reperfusion renal biopsies and Ingenuity®Pathway Analysis (IPA®, QIAGEN, USA) was used to identify differentially regulated pathways in 23 DBD and 16 DCD grafts (Supplemental Table 3). All biopsies were collected after

static cold storage, and prior to reperfusion. Biopsies were either immediately snap frozen in liquid nitrogen or stabilized in RNAlater.[5,6] Samples were all stored at −80°C. Total RNA was isolated from renal tissues using RNAzol (Campro Scientific, Vennendaal, The Netherlands)[5] or using TRI®Reagent according to the manufacturer's instructions (Invitrogen, UK),[6] cleaned and DNase treated (RNA Clean & Concentration, #R1015, Zymo Research, USA) then stored at −80°C for further analysis. RNA integrity was determined (RNA Nano kit and 2100 BioAnalyzer, #5067–1511, Agilent Technologies, Inc. USA) and samples with RIN>6.0 were used for further analysis. Briefly, total RNA was used to create libraries using ribosomal depletion (TruSeq Stranded Total RNA Ribo-Zero H/M/R Gold, Illumina). Libraries were further assessed by Qubit® (Life Technologies, Inc. USA) and Bioanalyser (High Sensitivity DNA kit [#5067–4626, Agilent Technologies, Inc., USA]). Libraries were sequenced on a NextSeq500 (Illumina) using a paired-end 2 × 75 bp run. Detailed methods and analysis approaches were described previously.[6] Raw count data were transformed to log2 scale to normalize expression counts. Multiple testing correction was performed using the Benjamin–Hochberg approach to control false discovery rate (FDR) at 10% (FDR ≤ 0.1 was considered significant). Differentially expressed gene targets were analyzed using Ingenuity®Pathway Analysis (IPA®, QIAGEN, USA).

Statistical analysis

STATA/SE version 12.0 (StataCorp, Texas, USA) and IBM SPSS Statistics 23.0 (Amsterdam, The Netherlands) were used for statistical analysis. Comparisons between groups were performed using the Mann-Whitney rank test and Kruskal-Wallis test for non-parametric data, independent t-test for normal-distributed data, and the Chi-Square test for categorical data. Cox proportional hazards models, censored for early graft loss (defined as functional graft loss within 90 days after transplantation) and recipient death, were used to evaluate differences in impact of DGF on 10-year graft survival. The model adjusted for donor/recipient age and sex, and cold ischemic period. An interaction (Wald) test was used to test the differences between the two models in DCD and DBD grafts. Factors associated with DGF were identified by multivariate regression analysis. The model included all variables with a p-value <0.10 in the univariate analysis. P-values <0.05 were considered statistically significant.

Acknowledgements

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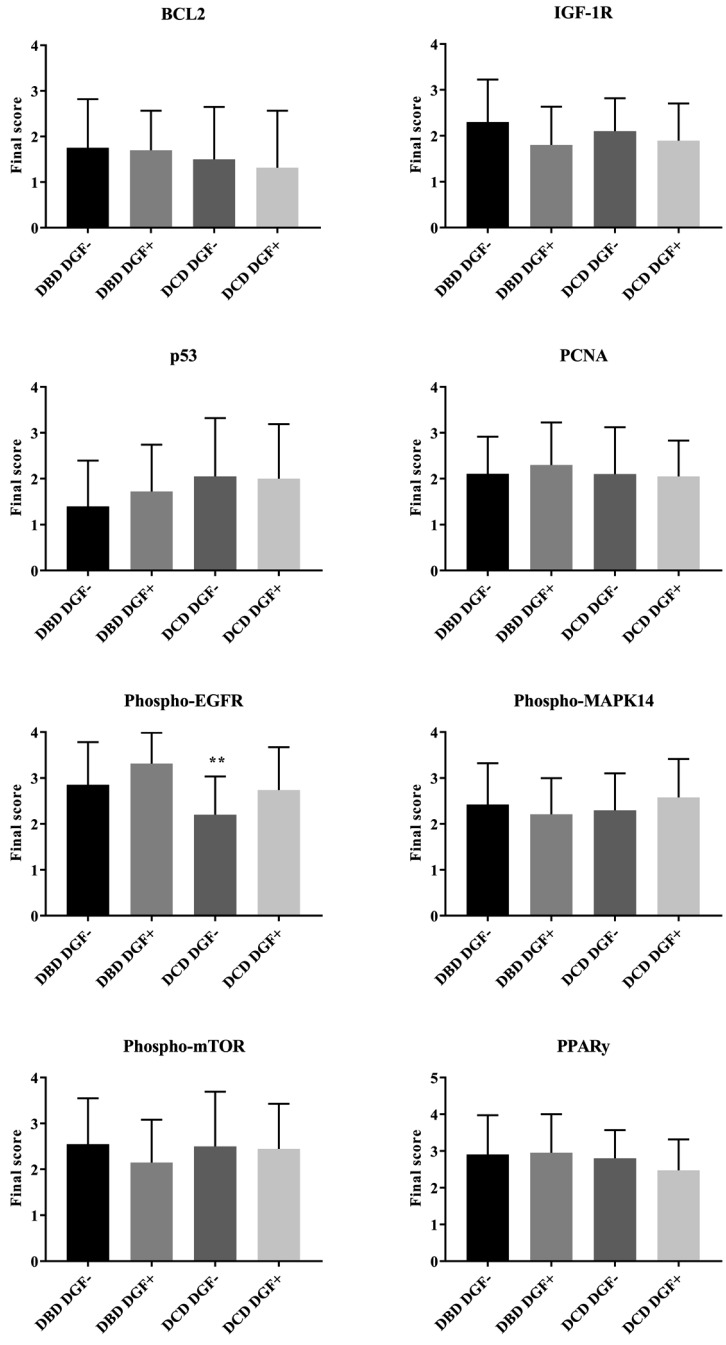
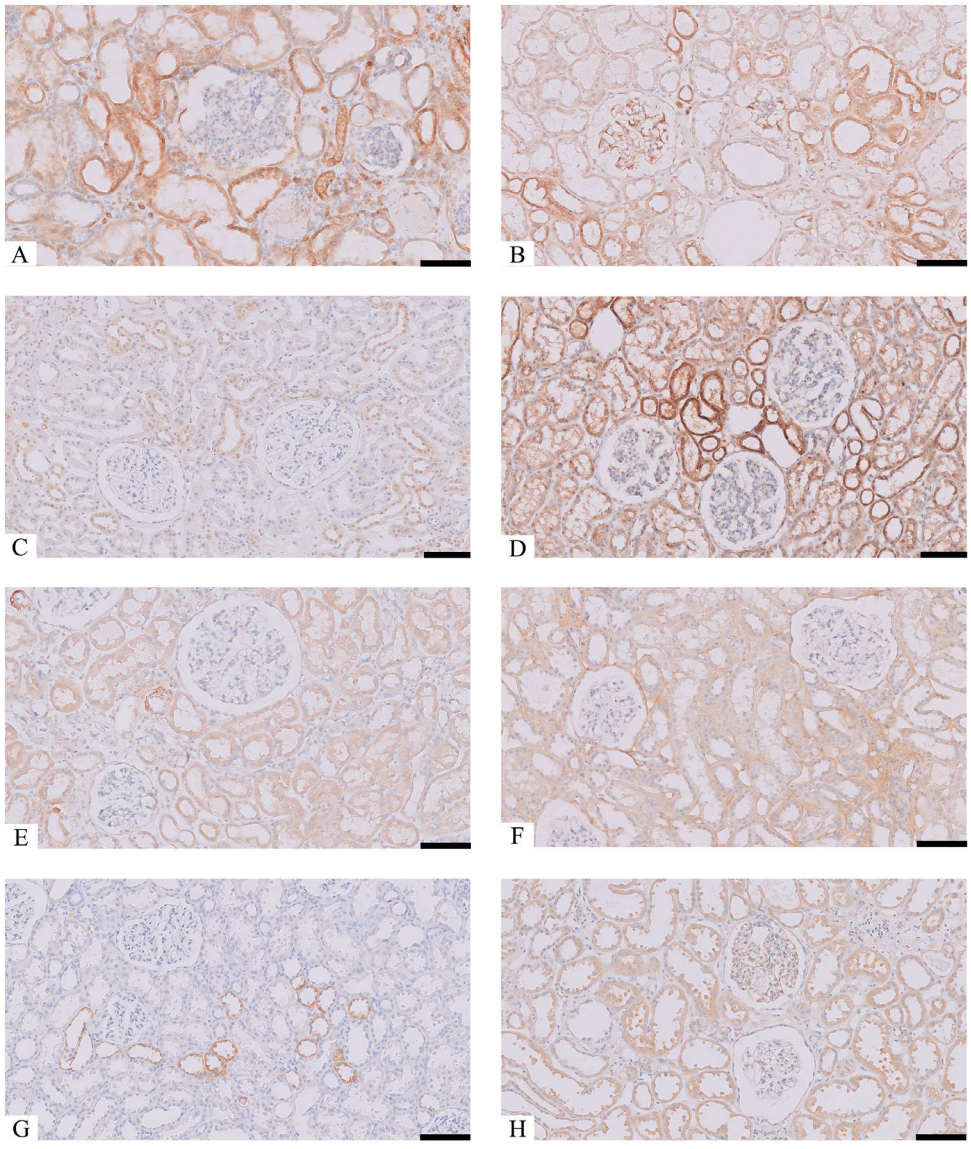
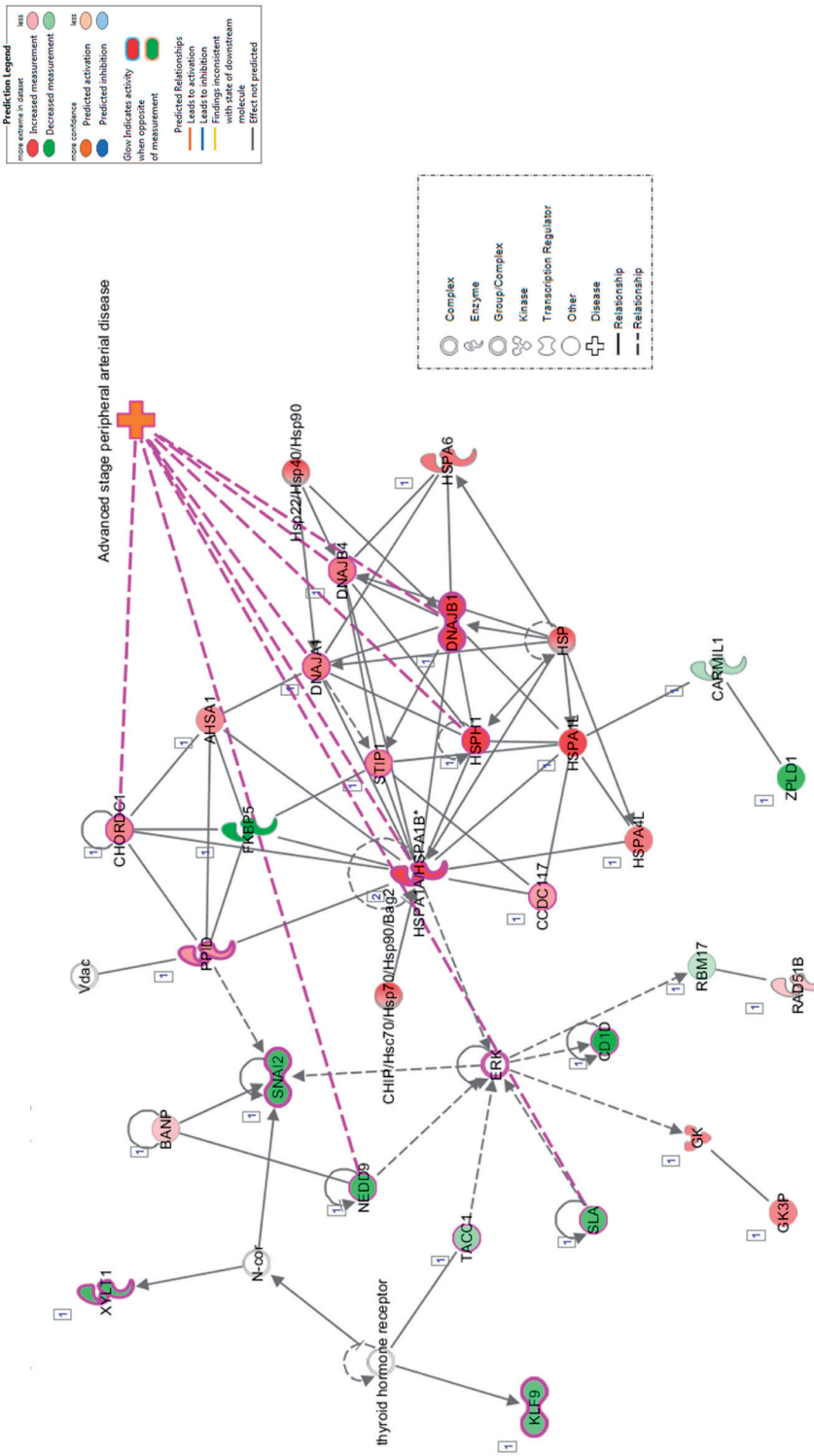


Supplemental Figure 1. Immunohistochemical scoring of pre-reperfusion kidney biopsies. Bars represent mean ± standard deviation.

Expression of BCL2 (Kruskal-Wallis test p = 0.653), IGF-1R (p = 0.340), p53 (p = 0.268), PCNA (p = 0.846), phospho-MAPK14 (p = 0.510), phospho-mTOR (0.554), PPARγ (p = 0.350) did not differ between groups. Expression of phospho-EGFR was lower in DCD grafts without DGF (p = 0.002).



Supplemental Figure 2. Immunohistochemical staining of pre-reperfusion kidney biopsies for factors associated with tumor resilience. Bars represent 100µm.
A = BCL-2; B = IGF-1R; C = p53; D = PCNA; E = phospho-EGFR; F = phospho-MAPK14; G = phospho-mTOR; H = PPARγ.



Supplemental Figure 3. Illustration of Ingenuity Pathway Analysis (IPA).
Strong influences were found on pathways collectively labelled by IPA as "cardio-vascular diseases". The network annotated as "advanced stage peripheral artery disease" constitutes the most differentially upregulated network (p-value <0.001). This network is dominated by members of the heat shock protein family.

Supplemental Table 1. Patient and transplant characteristics of the biopsies used for immunohistochemical evaluation.

		DBD DGF - n = 20	DBD DGF + n = 20	DCD DGF – n = 20	DCD DGF + n = 19	p-value
Donor	Age (years)	55 [49-63]	58 [44-64]	61 [52-64]	63 [52-65]	0.53
	Sex (% male)	11 (55%)	9 (45%)	12 (60%)	11 (58%)	0.79
Recipient	Age (years)	58 [46-69]	54 [44-67]	65 [54-69]	55 [46-73]	0.42
	Sex (% male)	10 (50%)	10 (50%)	16 (80%)	15 (79%)	0.06
Transplant	First warm ischemic period (min.)	NA	NA	14 [12-17]	16 [13-18]	0.28
	Cold ischemic period (hours)	10.7 [8.6–14.0]	14.9 [8.9–18.5]	11.7 [10.3–13.9]	12.1 [10.2–15.8]	0.31
	Graft anastomosis time (min.)	29 [22-33]	33 [28-38]	32 [27-37]	31 [23-38]	0.36
	Number of dialysis after transplantation	NA	4 [2-5]	NA	5 [3-7]	0.09

Data are presented as number (%) or as median [25 and 75 IQR]. NA: not applicable.

Supplemental Table 2. Details of antibodies used in this study.

Primary Antibody	Clone	Source	Retrieval	Dilution	Manufacturer	Catalog no.
BCL2	124	Monoclonal mouse	Tris EDTA	1:300	Dako	M0887
IGF-1R	3G5C1	Monoclonal mouse	Citrate	1:1000	ThermoFisher Scientific	MA5-15354
P53	DO-7	Monoclonal mouse	Tris EDTA	1:50	ThermoFisher Scientific	MA5-12557
PCNA	PC10	Monoclonal mouse	Citrate	1:300	ThermoFisher Scientific	13-3900
Phospho-EGFR	S.684.2	Monoclonal mouse	Tris EDTA	1:900	ThermoFisher Scientific	MA-15199
Phospho-MAPK14	-	Polyclonal rabbit	Citrate	1:300	Merck	SAB4300201
Phospho-mTOR	Ser 2481	Monoclonal mouse	Citrate	1:100	SantaCruz Biotechnology	Sc-293089
PPARγ	-	Polyclonal rabbit	Citrate	1:300	Bio-Rad	AHP1461

Supplemental Table 3. Patient and transplant characteristics of the biopsies used for gene expression profiles and Ingenuity Pathway Analysis.

		DBD n = 23	DCD n = 16	p-value
Donor	Age (years)	54.2 (15.7)	47.8 (15.8)	0.22
	Sex (% male)	11 (47.8%)	7 (43.8%)	0.70
Recipient	Age (years)	53.7 (12.9)	58.3 (8.3)	0.23
	Sex (% male)	12 (52.2%)	11 (68.8%)	0.38
Transplant	Cold ischemic period (hours)	12.5 [9.4–16.8]	11.6 [8.8–15.3]	0.51
	Graft anastomosis time (min.)	31.5 [24.8–33.5]	28.5 [24.0–30.8]	0.40
	Delayed graft function (% yes)	7 (30.4%)	12 (75.0%)	0.02

Data are presented as mean ± standard deviation (SD) or as number (%) or as median [25 and 75 IQR].

Supplemental Table 5. Multivariate analysis (Odds Ratio (95% CI)): risk factors associated with DGF.

	DBD	DCD
Donor age	1.012 (1.005–1.019)**	1.006 (0.999–1.012)
Donor last creatinine (μmol/L)	1.008 (1.006–1.010)**	1.000 (0.996–1.003)
Mismatch HLA-DR	1.215 (1.036–1.426)*	1.168 (0.992–1.357)
First warm ischemic period (min.)	NA	1.017 (1.004–1.031)*
Cold ischemic period (hours)	1.035 (1.021–1.049)**	1.019 (1.002–1.036)*
Graft anastomosis time (min.)	1.017 (1.010–1.024)**	0.996 (0.989–1.003)

*p<0.05; ** p<0.005, NA: not applicable.

Supplemental Table 4. Baseline patient and transplant characteristics.

		DBD n = 3744 (56.4%)	DCD n = 2891 (43.6%)	p-value
Donor	Age (years)	50.0 (15.0)	49.6 (15.0)	0.261
	Sex (% male)	1783 (47.6%)	1682 (58.2%)	< 0.001
	Height (cm)	173.0 (9.9)	175.0 (10.3)	< 0.001
	Weight (kg)	76.0 (15.6)	77.9 (16.7)	< 0.001
	BMI (kg/m^2)	25.3 (4.3)	25.3 (4.6)	0.648
	Last creatinine (µmol/L)	70.7 [56.0–91.0]	67.0 [53.0–83.0]	< 0.001
	Cause of death			< 0.001
	- Trauma	736 (19.7%)	806 (27.9%)	
	- Stroke	2241 (59.9%)	1123 (38.8%)	
	- Cardiac arrest	124 (3.3%)	418 (14.5%)	
	- Other	643 (17.2%)	544 (18.8%)	
	Hypertension (% yes)	984 (30.1%)	579 (21.1%)	< 0.001
	Diabetes (% yes)	168 (6.2%)	149 (5.7%)	0.407
	Smoking (% yes)	1760 (51.4%)	1378 (50.6%)	0.492
Recipient	Age (years)	52.1 (14.6)	54.1 (13.3)	< 0.001
	Sex (% male)	2170 (58.0%)	1806 (62.5%)	< 0.001
	Height (cm)	170.7 (10.2)	171.8 (10.3)	< 0.001
	Weight (kg)	73.9 (15.4)	76.7 (15.4)	< 0.001
	BMI (kg/m^2)	25.3 (4.5)	26.0 (4.4)	< 0.001
	Pre-emptive (% yes)	62 (1.7%)	57 (2.0%)	0.333
	Panel reactive antibodies			< 0.001
	- PRA <6%	3172 (84.7%)	2640 (91.3%)	
	- PRA ≥6 and <85	496 (13.3%)	235 (8.1%)	
	- PRA ≥85	75 (2.0%)	16 (0.6%)	
	Mismatches			
	HLA-DR 0	1558 (41.7%)	922 (32.1%)	< 0.001
	1	1883 (50.4%)	1718 (59.8%)	< 0.001
	2	292 (7.8%)	231 (8.0%)	< 0.001
	HLA-A 0	1451 (38.9%)	878 (30.4%)	
	1	1828 (49.0%)	1577 (54.7%)	
	2	455 (12.2%)	429 (14.9%)	
	HLA-B 0	978 (26.2%)	476 (16.5%)	
	1	1873 (50.2%)	1706 (59.2%)	
	2	883 (23.6%)	702 (24. 3%)	
Transplant	First warm ischemic period (min.)	NA	17.0 [14.0–21.0]	NA
	Cold ischemic period (hours)	17.0 [13.1 – 22.0]	16.0 [12.6 – 20.1]	< 0.001
	Graft anastomosis time (min.)	34.0 [26.0 – 41.0]	32.0 [26.0 – 40.0]	0.003

Data are presented as mean (± standard deviation) or as number (%) or as median [25 and 75 IQR]. NA: not applicable.

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PART II

Studying the pathophysiology of metabolic failure in ischemia-reperfusion injury

Chapter 5

Clinical ischemia-reperfusion injury: driven by reductive rather than oxidative stress?

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Submitted.

Abstract

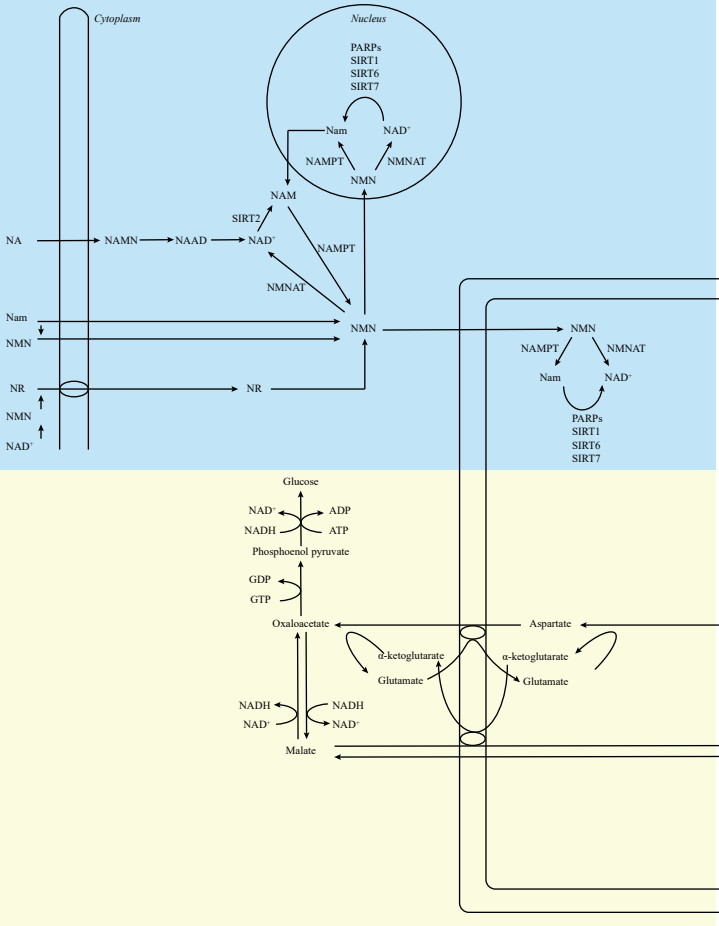
In clinical practice, ischemia-reperfusion injury (I/R injury) remains a major contributor to organ damage following transient hypoxic insults. Although numerous interventions have been suggested to be effective and to reduce I/R injury in preclinical models, none of these therapies have been successfully translated to the clinical setting. In the context of this 'translational gap' we have focused on recent clinical observations that have reported on a discriminatory metabolic signature of renal I/R injury, that in the clinical context manifested itself as delayed graft function after kidney transplantation. This signature points to the presence of a persistent postreperfusion metabolic paralysis, tricarboxylic acid cycle defects and a compensatory activation of catabolic routes. Against this background, the picture emerges that clinical I/R injury might be actually driven by reductive stress. In this perspective article, we have evaluated on the processes contributing to reductive stress in the context of I/R injury and try to provide better insight in potential clinical therapeutic strategies that may be helpful in restoring the redox balance.

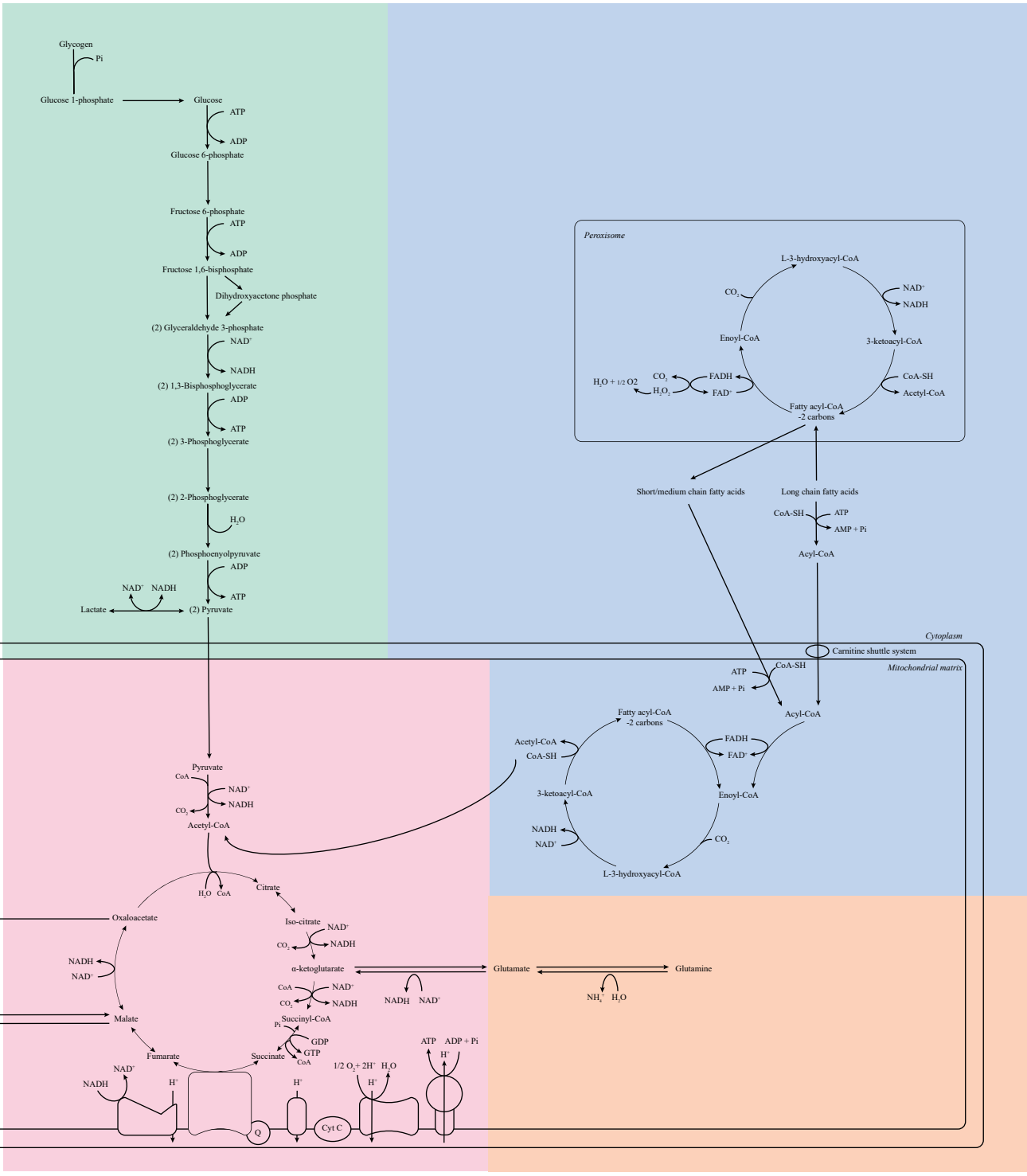
Introduction

Ischemia-reperfusion injury (I/R injury) describes the increase of tissue injury following oxygenated reperfusion with blood of a previously ischemic tissue or organ after restoration of normothermic conditions. Clinically, I/R injury is a contributor to organ damage following transient hypoxic insults, i.e. myocardial infarction and stroke, as well as for the non-immunologic graft damage after transplantation. Although numerous strategies to prevent or alleviate I/R injury have been reported, with some of them effective in animal models, unfortunately, none of these therapies have been successfully translated to the clinical setting.[1-3]

Using human kidney transplantation as an example of predicted ischemia and reperfusion, and delayed graft function as its clinical read-out, we have systematically studies the mechanisms reported to be of relevance in I/R injury.[4] In a number of studies, no primary role has been observed of oxidative damage, complement activation or inflammation [5-7], instead, strong indications have been found for the metabolic origin of I/R injury. Since similar conclusions were reached by other groups [8,9], we considered an attempt to map the metabolome of clinical I/R injury as relevant. The results of this evaluation have identified a distinct discriminative metabolic signature for I/R injury.[4] This signature points at the presence of a persistent postreperfusion metabolic paralysis as indicated by persistent (>30 minutes) post-reperfusion ATP/GTP catabolism, tricarboxylic acid (TCA) cycle defects, and a comprehensive activation of catabolic routes. Against this background, the picture emerges that whilst I/R injury may be triggered by oxidative damage[9], clinical I/R injury is actually driven by reductive stress. To be more specific, the compensatory catabolic state initiated by high-energy phosphate depletion results in a parallel production of reducing equivalents, i.e. NADH and $FADH_2$. Whilst these equivalents are normally oxidized through the electron transport chain (ETC) (Complex I and II, respectively) or by cytosolic LDH (glycolysis), the excess reductive load and apparent I/R injury -related defects in the oxidative routes may result in accumulation of reducing equivalents and thus cause reductive stress (Figure 1). In this article, we have evaluated the processes described that will contribute to reductive stress in the context of I/R injury and provide better insight in potential clinical therapeutic strategies that can be helpful in restoring the redox balance.

Figure 1. NADH NAD metabolism.




Glycogen
Pi
Glucose 1-phosphate
Glucose
ATP
ADP
Glucose 6-phosphate
Fructose 6-phosphate
ATP
ADP
Fructose 1,6-bisphosphate
Dihydroxyacetone phosphate
(2) Glyceraldehyde 3-phosphate
NAD+
NADH
(2) 1,3-Bisphosphoglycerate
ADP
ATP
(2) 3-Phosphoglycerate
(2) 2-Phosphoglycerate
H2O
(2) Phosphoenyolpyruvate
ADP
ATP
NAD+ NADH
Lactate
(2) Pyruvate
Peroxisome
L-3-hydroxyacyl-CoA
CO2
NAD+
NADH
Enoyl-CoA
3-ketoacyl-CoA
H2O + 1/2 O2
CO2
H2O2
FADH
FAD+
CoA-SH
Acetyl-CoA
Fatty acyl-CoA
-2 carbons
Short/medium chain fatty acids
Long chain fatty acids
CoA-SH
ATP
AMP + Pi
Acyl-CoA
Cytoplasm
Carnitine shuttle system
Mitochondrial matrix
ATP
CoA-SH
AMP + Pi
Acyl-CoA
Fatty acyl-CoA
-2 carbons
Acetyl-CoA
CoA-SH
FADH
FAD+
3-ketoacyl-CoA
Enoyl-CoA
NADH
NAD+
CO2
L-3-hydroxyacyl-CoA
Pyruvate
CoA
NAD+
NADH
CO2
Acetyl-CoA
H2O CoA
Citrate
Oxaloacetate
Iso-citrate
NAD+
CO2
NADH
NADH
NAD+
α-ketoglutarate
Glutamate
Glutamine
CoA
NAD+
CO2
NADH
NADH NAD+
NH4+ H2O
Malate
Succinyl-CoA
Pi
GDP
GTP
CoA
Fumarate
Succinate
ATP ADP + Pi
H+
NAD+
NADH
H+
H+
1/2 O2 + 2H+ H2O
H+
Q
Cyt C

Excessive NADH and $FADH_2$ formation in ischemia-reperfusion injury

The metabolome of renal I/R injury (clinically expressed as delayed graft function) is summarized in Table 1. This I/R injury (delayed graft function-specific) metabolome is hallmarked by a comprehensive activation of oxidation routes including glycolysis, fatty acid β-oxidation, glutaminolysis, and oxidation of branched chain amino acids. The latter presumably reflecting activation of autophagy.[4] The molecular clues for this catabolic signature are illustrated in Table 1. Oxidation of these 'fuel' sources is paralleled by a concomitant production of reducing equivalents (NADH and $FADH_2$) which attribute to an increase of the reductive load.

Disturbed NAD^+ regeneration and TCA cycle defects

Within the physiological context, redox-neutrality is maintained by oxidation of NADH and $FADH_2$ in the mitochondrial ETC (donating their electrons to complex I and II, respectively), and NADH by cytosolic LDH (oxidation of pyruvate to lactate

Table 1. Metabolic signature of clinical renal ischemia reperfusion injury (delayed graft function) based on arteriovenous differences (IN: uptake by the graft from arterial blood, OUT: release by the graft in the venous effluent) and tissue biopsies (DOWN and UP: tissue contents lower respectively higher than in control kidneys without ischemia reperfusion injury). * from glutaminolysis; # intermediates of branched chain amino acid oxidation.

Process	Flag		
Impaired ATP/GTP re-synthesis	(Hypo) Xanthine	OUT	
	Tissue phospho-creatine	DOWN	
β-oxidation	Medium chain fatty acid	IN	$NAD^+ \rightarrow NADH$
	Tissue ß-hydroxybutyric acid	UP	$FAD \rightarrow FADH_2$
Activated glycolysis	Pyruvate	OUT	$2\ NAD^+ \rightarrow 2\ NADH$
	Phosposerine	OUT	$NADH^+ \rightarrow NAD^+$
	Lactate	OUT	
	Alanine	OUT	
Glutaminolysis / Impaired malate-glutamate shuttle	Glutamate	OUT	$NAD^+ \rightarrow NADH^*$
	Tissue glutamate	DOWN	
	Aspartate	OUT	
Autophagia (branched chain amino acids)	Isovalerylcarnitine #	OUT	$NAD^+ \rightarrow NADH$
	Butyrylcarnitine #	OUT	$NAD^+ \rightarrow NADH$
TCA cycle entry defect	Acetylcarnitine	OUT	
	Tissue acetylcarnitine	UP	
TCA cycle defect	α-ketoglutarate	OUT	$NAD^+ \rightarrow NADH$
	Tissue succinate	DOWN	

(glycolysis)).[10, 11] An increased blood lactate/pyruvate ratio in grafts with delayed graft function signals an impaired redox status. While this imbalance may directly link to the comprehensive activation of catabolic pathways (increased reductive load), compelling evidence also exists for defect(s) in the mitochondrial respiratory chain.
Multiple studies have reported a defect or decreased activation of particularly complex I in the context of I/R injury.[12-15] This defect might be (partially compensated) by the recruitment of cytosolic NADH through the malate-aspartate shuttle and LDH. Yet, the impaired lactate/pyruvate ratio from renal grafts with I/R injury implies a reductive load that exceeds the capacity of LDH [4, 16], whereas post-reperfusion aspartate release points to interruption of the malate-aspartate shuttle.

System overload

As a consequence of the findings mentioned above, it appears that I/R injury may be driven by a 'system overload' caused by a wider activation of catabolic pathways, a concomitant reductive burden (Table 1), and an impairment of the oxidative machinery that results in an impaired reduction to NAD^+ and FAD; all in combination, resulting in a condition of profound reductive stress. The existence of this system overload is best illustrated by the release of acetyl-carnitine and α-ketoglutarate from kidney grafts suffering from I/R injury. Acetyl-carnitine and isovaleryl-carnitine release imply acyl production that exceeds the oxidative capacity of the TCA cycle. Specific release of α-ketoglutarate but not its TCA cycle oxidation products (as indicated by low tissue succinate levels) implies a defect at the level of α-ketoglutarate dehydrogenase, and to an increased production of α-ketoglutarate (glutaminolysis, oxidation of branched-chain amino acids). Activity of the α-ketoglutarate dehydrogenase complex relies on specific cofactors such as TTP, CoA, lipoate, and importantly FAD and NAD^+ as the final reducing equivalent (17). Critical reliance on these co-factors implies a vicious circle that sustains a situation of reductive stress.

Potential therapeutic strategies

Based on these considerations, two potential therapeutic strategies for clinical I/R injury can be considered. First, reducing the reductive burden (NADH and $FADH_2$ production), and second, boosting NAD^+ levels, either by supplementation of NAD^+ precursors or by activation of enzymes regulating NAD^+ synthesis.

The first strategy is based on the observation that the metabolome of early I/R injury is characterized by futile substrate fluxes. In fact, data show that comprehensive activation

of catabolic routes (possibly in response to the exhaustion of high energy phosphates) exceeds the oxidative capacity of the TCA cycle and LDH. Consequently, a logical strategy may be to reduce the futile carbon-fluxes. Such a strategy is supported by experimental evidence showing that removal of fatty acids, lactate and insulin from the perfusate restores the [NAD^+]/[NADH]ratio and facilitates ex-vivo recovery of mouse hearts.[18] On theoretical grounds, it could be speculated that inhibition of β-oxidation will be the most effective strategy in the context of renal I/R injury since β-oxidation associates with the highest reductive load. In this context, a targetable candidate is L-3-hydroxyacyl-CoA dehydrogenase which is inhibited by acetoacetyl-CoA (intramitochondrial inhibition) or perfluorodecanoic acid (intraperoxisomal inhibition).[19] Other, β-oxidation independent strategies include the use of glutaminase inhibitors (glutaminolysis) and glycolysis inhibitors (for the inhibition of excessive glycolysis).[20, 21]

The second and non-exclusive strategy is restoration of NAD^+ levels by administration of NAD^+ precursors, or by activation of NAD^+ biosynthetic enzymes. Although supplementation of various NAD^+ precursors (nicotinamide riboside with or without pterostilbene) has been found effective in the clinical setting (i.e. healthy individuals and patients suffering from acute kidney injury) [22-25], its translation to the I/R injury setting can be considered as challenging. In fact, clinical trial data indicate that augmentation of cellular NAD^+ levels only occur hours or even days after its oral administration. [22-25] Given that the metabolic collapse in I/R injury occurs within minutes of reperfusion, it is unlikely that these indirect (time and energy requiring 'booster' strategies will effectively improve the redox balance in the acute phase of I/R injury, although it cannot be excluded that the strategy may accelerate metabolic recovery. Moreover, NAD^+ boosting therapies might be considered as a prevention strategy for an expected I/R injury, such as in organ transplantation or in planned major surgery with arterial cross-clamping as well as cardiothoracic surgery. Similar considerations also apply to the pharmacological induction or activation of NAD^+ biosynthetic enzymes.[11, 26] Reportedly, NAD^+ boosting can be achieved by overexpression of the NAD^+-synthetic enzymes NAMPT and NMNAT, or by direct enzyme-activation with use of pharmacologic compounds such as P7C3 and SBI-797812.[11, 26-31] However, considering the prolonged response times and the reliance on agile gene translation and transcription, it is unlikely that this strategy can be used as a rescue therapy for I/R injury.

Conclusions

Whilst oxidative damage is widely considered as the key driver of I/R injury, clinical observations suggest that a post-reperfusion metabolic paralysis caused by reductive stress as the actual effector mechanism of I/R injury. The apparent inability of cells to

generate high energy phosphates results in exhaustion of the ATP/GTP pool, causing a cataplexic state that interferes with cellular homeostasis. Depletion of the high energy pool triggers a comprehensive compensatory activation of catabolic pathways (i.e. glycolysis, fatty acid β-oxidation, autophagia and glutaminolysis) in an apparent more or less futile attempt to drive ATP generation. Activation of these catabolic pathways results in a concomitant increased reduction of NAD^+ to NADH and FAD to $FADH_2$, that cannot be compensated by the mitochondrial oxidative machinery. Based on these observations we suggest to focus on strategies that aim to alleviate clinical I/R injury by inhibiting processes that excessively produce NADH and $FADH_2$ (e.g. medium chain fatty acid β-oxidation) or by promoting preventive exogenous replenishment of the NAD^+ pool to restore the redox balance.

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Chapter 6

Preclinical models versus clinical renal ischemia reperfusion injury: a systematic review based on metabolic signatures.

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Abstract

Despite decennia of research and numerous successful interventions in the preclinical setting, renal ischemia reperfusion (I/R) injury remains a major problem in clinical practice, pointing towards a translational gap. Recently, two clinical studies on renal I/R injury (manifested either as acute kidney injury or as delayed graft function), identified metabolic derailment as a key driver of renal I/R injury. It was reasoned that these unambiguous metabolic findings enable direct alignment of clinical with preclinical data, thereby providing the opportunity to elaborate potential translational hurdles between preclinical research and the clinical context. A systematic review of studies that reported metabolic data in the context of renal IR was performed according to the PRISMA guidelines. The search (December 2020) identified thirty-five heterogeneous preclinical studies. The applied methodologies were compared, and metabolic outcomes were semi-quantified and aligned with the clinical data. This review identifies profound methodological challenges, such as the definition of I/R injury, the follow-up time and sampling techniques, as well as shortcomings in the reported metabolic information. In light of these findings, recommendations are provided in order to improve the translatability of preclinical models of renal I/R injury.

Introduction

Ischemia reperfusion (IR) injury describes the paradoxical increase in tissue injury following reperfusion of transiently ischemic organs. I/R injury contributes significantly to graft damage in the context of organ transplantation. Unfortunately, despite decades of research and numerous preclinical successes, no intervention to date successfully reduced clinical I/R injury.[1,2]

The notable contrast between preclinical successes and consistent clinical failures points towards a profound translational gap in the understanding of I/R injury. Independently of each other, two recent clinical studies implied metabolic failure as the primary effector mechanism of renal I/R injury. To be specific, these studies concluded that both delayed graft function (DGF) in the context of kidney transplantation, as well as acute kidney injury (AKI) in the context of major cardiac surgery, associate with profound, transient post-reperfusion metabolic defects such as, in the case of DGF, post-reperfusion normoxic glycolysis and persistent post-reperfusion ATP catabolism (further details are summarized in Table 1 and Figure 1).[3,4]

We reasoned that these unambiguous observations for clinical renal I/R injury provide the opportunity to validate reported preclinical models. Therefore, we performed a systematic literature review to identify studies that report on metabolic aspects of experimental renal I/R injury. Methodological aspects and reported metabolic observations in these studies were aligned with the clinical context in an attempt to map parallels and dissimilarities between preclinical models and clinical context.

Table 1. Two recent clinical studies reporting renal metabolic data after ischemia and reperfusion (IR) resulting in acute kidney injury (AKI) or delayed graft function (DGF)[3,4].

Article	Sample timing	IR injury definition	Results on metabolome
Legouis et al. 2020[3]	Blood: twice at a 30-min interval. Control group: 4-6 h post-IR; AKI group: 2-6 days post-IR.	AKI: KDIGO criteria	In patients experiencing AKI: switch from net renal lactate uptake to net renal lactate release, a decrease in net renal glucose release compared to that in the control group.
Lindeman et al. 2020[4]	Blood: renal artery: 0, 10, 30 min post-IR; renal vein: 0.5, 3, 5, 10, 20, 30 min post-IR Tissue: post-ischemia and 45 min post-IR	DGF: recipient requires dialysis in first week(s) post-transplantation, excluding dialysis for hypervolemia, hyperkalemia or hyperphosphatemia.	Grafts manifesting future DGF: post-reperfusion ATP/GTP catabolism (significantly impaired phosphocreatine recovery and significant persistent (hypo)xanthine production). Failing high-energy phosphate recovery occurred despite activated glycolysis, fatty-acid oxidation, glutaminolysis and autophagia, and related to a defect at the level of the oxoglutarate dehydrogenase complex in the Krebs cycle.

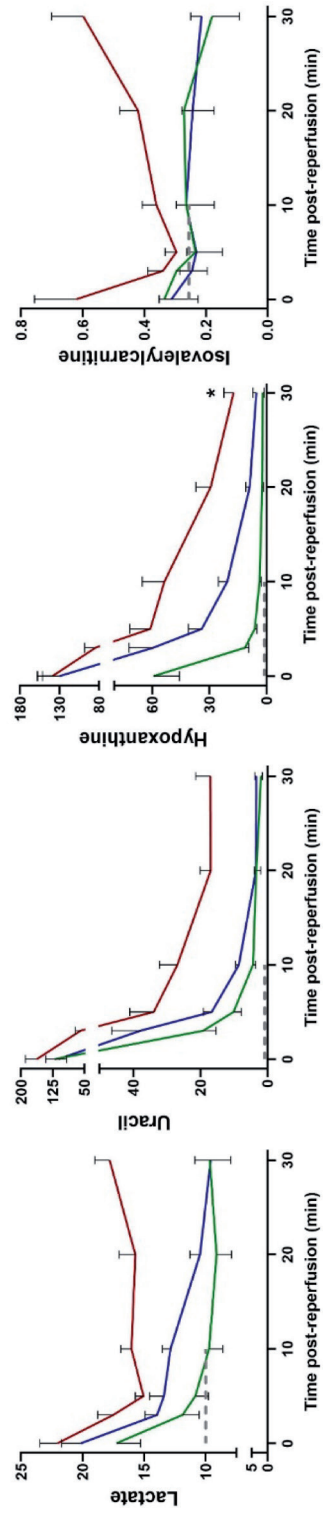

Figure 1. Illustration of the contrasting post-reperfusion metabolic responses of kidney donor grafts with Delayed Graft Function (DGF, IR injury, red curve) and grafts recovering without IR injury (no DGF) (green curve: living donor graft (intermediate ischemic period), blue curve: deceased donor graft (prolonged ischemic period)). The dashed line reflects the normal, non-ischemic kidney. Figures adapted from Lindeman et al.[4]. Curves represent renal vein levels of lactate (glycolysis), uracil (cellular damage)[5], hypoxanthine (ATP catabolism, metabolic incompetence), and isovalerylcarnitine (intermediate of branched chain amino acid oxidation (autophagia)). *: decrease at end of measurement window may reflect depletion of ATP pool.

Methods

Systematic searches (see Supporting Information) were performed in PubMed, EMBASE and Web of Science to identify preclinical studies reporting metabolic data following renal IR in the context of DGF and AKI. Articles were selected following recommended procedures described by PRISMA guidelines.[6] Two authors independently assessed titles and abstracts for eligibility. Full texts were consulted if it was unclear whether inclusion criteria were met.

Results

The literature searches are summarized in two diagrams (Supporting Figure 1). Thirty-four studies were selected based on the predefined inclusion criteria. One extra study (unidentified in both searches) was included. Thus, thirty-five preclinical studies were included, of which seventeen explored renal IR in rats, ten in mice, one in both rats and mice (included as two separate studies), four in pigs, and two in dogs. Almost all studies were performed in homogeneous populations of particularly young, mostly male, and healthy animals. The dog studies included an explicitly heterogeneous study population of mongrel dogs. Details of the methodology and results of the included reports are summarized in Table 2 and 3.

Measures of renal I/R injury

Although some variability exists for the clinical definitions of AKI and DGF (Table 4), definitions are essentially functional and outcome-centered.[40,41] The two landmark clinical studies that report the metabolome of respectively AKI and DGF both used 'conservative' definitions.[3,4] The diagnosis of AKI was based on KDIGO criteria (Table 4)[41], and DGF was defined as the need for dialysis for at least the first week after transplantation.[40] Partial or full functional recovery is an inherent aspect of both clinical definitions.

The majority (23/35) of the included preclinical studies used post-reperfusion serum creatinine levels as a functional measure of I/R injury (Table 3). In contrast to clinical definitions of AKI, no predefined thresholds for the diagnosis of I/R injury were considered. None of the preclinical studies included the need for (transient) renal replacement therapy as outcome measure. A third of the studies (11/35) reported histological grading as surrogate outcome parameter (Table 3). Other parameters used were diverse: e.g. body and/or kidney weight, and expression of the damage markers kidney injury molecule-1 (KIM-1) and/or neutrophil gelatinase-associated lipocalin (NGAL).

The dynamics of recovery is an inherent aspect of the clinical diagnosis of renal I/R injury (Table 4), but could not be properly addressed in the majority of experimental studies

Table 2. Methodological details reported in the publications included by the systematic search.

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Rat										
Andrianova et al. 2020[7]	Rat	OutbredWistar rats Male	3-4 months 300-400 g	Clamping Right nephrectomy, clamping of left renal vascular bundle for 40 min.	-	40	Blood (carotid artery)	Blood: 48 h post-IR	Blood: intact control	Blood metabolomics: FIA-MS/MS Serum levels urea & creatinine: AU480 Chemistry System
Choi et al. 2019[8]	Rat	Sprague-Dawley rats Male	3-4 months 410-450 g	Surgery 20 min of cardiac arrest through asphyxia, then resuscitation by cardiopulmonary bypass.	-	20	Tissue	Tissue: post-I and 30 min post-IR After 30 minutes of cardiopulmonary bypass resuscitation, rats were euthanized to harvest brain, heart, kidney, and liver tissue samples.	Tissue: intact control, no cardiac arrest, euthanized 7 min before harvesting tissues.	Tissue metabolomics: LC-MS/MS
Duran et al. 1990[9]	Rat	Sprague-Dawley rats Female	??? 240-312 g	Clamping Unilateral clamping of left renal artery for 1 h.	-	60	Blood (cardiac puncture) Tissue (cortex)	Blood, tissue: 3 and 24 h post-IR	Blood, tissue: "control" rats Blood (for BUN): prior to IR, from tail	Blood/tissue metabolomics: dansylation of amino acids and subsequent chromatography BUN: autoanalyzer technique
Gaudio et al. 1991[10]	Rat	Sprague-Dawley rats	??? 250 g	Clamping Clamping of aorta proximal to both renal arteries for 45 min.	-	45	Tissue	Tissue: 15 min or 2 h post-IR, measurements om proximal tubule suspensions.	Tissue: sham-operated rats	ATP content: HPLC
Huang et al. 2018[11]	Rat	Fisher F344 rats	??? 250-300 g	Clamping Unilateral clamping of left renal artery for 45 min.	-	45	Blood (???) Tissue (cortex)	Blood, tissue: 4 h and 24 h post-IR	Blood and tissue: contralateral kidney and kidneys from healthy control rats	Tissue metabolomics: ^{1}H-NMR & GCxGC-MS
Lan et al. 2016[12]	Rat	Sprague-Dawley rats Male	???	Clamping Right nephrectomy, clamping of left renal vascular pedicle for 45 min.	-	45	Blood (???) Tissue (cortex and outer stripe of outer medulla)	Blood: daily post-IR Tissue: 3, 7 and 14 days post-IR	Blood: prior to surgery Tissue: sham-operated rats	Tissue lactate and pyruvate levels: fluorimetry SCr: ???

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Legouis et al. 2020[3]	Rat	Sprague–Dawley rats Male	8-10 weeks ???	Clamping Right nephrectomy, clamping of left renal artery for 25 min.	-	25	Blood (left femoral artery, left femoral vein and renal vein)	Blood: 60 and 120 min post-IR	Blood: sham-operated rats	Gluconeogenesis (plasma D2-glucose enrichment after administration of D2-glucose) measurement in blood: GC-MS
Lindhardt et al. 2020[13]	Rat	Wistar rats Male	??? 205-290 g	Clamping Unilateral clamping of left renal artery for 40 min.	-	40	Blood (tail vein) Tissue imaging *in vivo* Urine (metabolic cage)	Blood, tissue (imaging *in vivo*): 24 h post-IR Urine: 24 h post-IR	Blood: no control Tissue imaging *in vivo*: contralateral kidney Urine: no control	Tissue metabolomics: *In vivo* ^{1}H and hyperpolarized ^{13}C MRI Urinary creatinine, BUN and SCr: COBAS 6000 device (Roche).
Liu et al. 2012[14]	Rat	Sragues-Dawley rats Male	Adult 175-225 g	Clamping Clamping of renal arteries for 45 min.	-	45	Blood (posterior orbital venous plexus) Tissue (cortex)	Blood: 2, 4, 6, 12, 24, 48, 72 and 96 h post-IR Tissue: 24 h and 96 h post-IR	Blood and tissue: sham-operated rats	Blood metabolomics: HPLC/MS
Nielsen et al. 2017a[15]	Rat	Wistar rats Male	??? 200-250 g	Clamping Unilateral clamping of left renal artery for 40 min.	-	40	Blood (arterial) Tissue Urine (metabolic cage)	Blood, tissue imaging *in vivo* and samples: 24 h post-IR Urine: 24 h post-IR	Blood: prior to surgery Tissue (imaging *in vivo* and samples): contralateral kidney Urine: 24 h prior to surgery	Tissue metabolomics: *In vivo* ^{1}H and hyperpolarized ^{13}C MRI
Nielsen et al. 2017b[16]	Rat	Wistar rats Male	??? 250-290 g	Clamping Unilateral clamping of left renal artery for 30 or 60 min.	-	30 or 60	Blood (??? before surgery and aorta post-IR) Tissue imaging *in vivo* Tissue samples (cortex)	Blood, tissue imaging *in vivo* and samples: 24 h post-IR	Blood: prior to surgery (24 h) and sham-operated rats Tissue: contralateral kidneys and sham-operated rats	Tissue metabolomics: *In vivo* ^{1}H and hyperpolarized ^{13}C MRI Tissue lactate levels: enzymatic assay BUN and SCr: COBAS 6000 device (Roche) Renal KIM-1 and NGAL expression: qPCR

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Nielsen et al. 2020[17]	Rat	Wistar rats Male	??? 200-245 g	Clamping (2 distinct procedures) Procedure 1: Unilateral clamping of left renal artery for 30 min. Procedure 2: Unilateral clamping of left renal artery for 20 or 40 min.	-	20, 30 or 40	Blood (teil vein) Tissue imaging *in vivo* Tissue samples (cortex and inner medulla)	Blood: directly before tissue imaging *in vivo* Tissue imaging *in vivo*: Procedure 1 – 2 and 60 min post-IR Procedure 2 – 1 and 7 days post-IR Tissue samples: 60 min post-IR and 7 days post-IR	Blood: no control Tissue (imaging *in vivo* and samples): contralateral kidney	Tissue metabolomics: *In vivo* ^{1}H and hyperpolarized ^{13}C MRI SCr: COBAS 6000 device (Roche)
Peto et al. 2018[18]	Rat	Crl:WI rats Male	??? 342.2 ± 29.5 g	Clamping Ligation of right renal artery and clamping of left renal vessels for 60 min. After WI, excision of right kidney and clamp removal from the left renal vessels.	-	60	Blood (left femoral artery) Tissue	Tissue: 120 min post-IR Blood: pre-I, post-I, 60 min post-IR and 120 min post-IR	Blood and tissue: prior to surgery and sham-operated rats	Blood acid-base parameters, glucose and electrolytes: EPOC portable blood analysis device
Serkova et al. 2005[19]	Rat	Lewis rats Male	??? 200-250 g	Transplantation After removal from living donors, kidneys were kept cold for 24 or 42 h. Implantation after removal of both kidneys from recipient.	24 or 42	???	Blood (???) Tissue	Blood: pre-IR, 24 h post-IR Tissue: pre-IR, post-I, 24 h post-IR	Blood and tissue: recipient's kidney and blood prior to nephrectomy and transplantation	Blood/tissue metabolomics: ^{1}H-NMR
Shen et al. 2017[20]	Rat	Sprague-Dawley rats Female	Adult 200-220 g	Clamping Clamping of renal pedicles for 30 min.	-	30	Tissue	Tissue: post-IR (reperfusion time unknown)	Tissue: sham-operated rats	Tissue metabolomics: GC/MS
Tani et al. 2019[21]	Rat	Sprague-Dawley rats Male	6 weeks ???	Clamping Unilateral clamping of left renal pedicle for 30 min.	-	30	Tissue	Tissue: 60 min after drug administration, post-I, and 30 min post-IR	Tissue: "vehicle treatment group", no surgery, 60 min after receiving 0.5 mL 0.5% methylcellulose.	Tissue purine nucleotide concentration: HPLC Tissue metabolomics: CE-ToFMS

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Trifillis et al. 1984[22]	Rat	Sprague-Dawley rats Male	??? 220-250 g	Clamping Clamping of aorta above left renal artery, below the right renal artery and superior mesenteric artery, as well as clamping right renal artery and vein for 15, 10, 60, 90 or 120 min.	-	5, 15, 30, 60, 90 or 120	Blood (aorta) Tissue	Blood: ??? Tissue: post-I, and 0.25, 1, 6, 24 or 48 h post-IR	Blood: ??? Tissue: control rats (no sham surgery)	Tissue ATP/ADP/AMP/lactate measurements: specific enzymatic methods coupled with NADH or NADPH Tissue Pi measurements: modification of Fiske and SubbaRow method SCr: Beckman creatinine analyzer II. BUN: Beckman urea nitrogen analyzer.
Varga et al. 2019[23]	Rat	Crl:WI rats Male	??? 301.6 ± 38.6 g	Clamping Unilateral clamping of left renal vessels for 45 min.	-	45	Blood (right femoral artery)	Blood: pre-I, and 30, 60 and 120 min post-IR	Blood: prior to surgery and sham-operated rats	Blood acid-base parameters, metabolites and electrolytes: EPOC portable blood analysis device
Mouse										
Beier et al. 2020[24]	Mouse	C57BL/6 mice Female	???	Clamping Unilateral clamping of renal pedicle for 28 min.		28	Tissue	Tissue: 24 h post-IR	Tissue: contralateral kidney	Tissue metabolomics: UPLC-MS
Chihanga et al. 201[82]5	Mouse	Swiss-Webster mice	??? 25-30g	Clamping Clamping of renal pedicles for 30 min.	-	30	Blood (inferior vena cava for SCr, cardiac puncture for NMR) Tissue Urine (metabolic cage)	Blood: 24 h post-IR Tissue: 24 h post-IR Urine: 3 days pre-IR and 24 h post-IR	Blood: control mice (pre-IR) Tissue: control mice (pre-IR) Urine: 3 days pre-IR	Blood/urine metabolomics: ^{1}H-NMR Urinary creatinine, SCr and urinary NGAL: spectroscopy

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Cho et al. 2017[26]	Mouse	C57BL/6 mice Male	9 weeks ???	Clamping Unilateral clamping of left renal pedicle for 45 min.	-	45	Blood (???) Tissue Urine (metabolic cage)	Blood: 24 h post-IR Tissue: 24 h post-IR Urine: 24 h post-IR	Blood, tissue and urine: sham-operated mice	Blood/tissue/urine metabolomics: HPLC-Q-ToF MS
*Chouchani et al. 2014[27]	Mouse	C57BL/6J mice Male	8-10 weeks ???	Clamping Unilateral clamping of one renal pedicle for 45 min.	-	45	Tissue	Tissue: post-I and 5 min post-IR	Tissue: ??? (normoxic controls)	Tissue metabolomics: LC-MS
Fujii et al. 201[92]8	Mouse	C57BL/6 Male	8 weeks ???	Clamping Unilateral clamping of left renal artery for 1, 10 and 40 min.	-	1, 10 and 40	Blood (???) Tissue Urine (???)	Tissue: after 1, 10, and 40 min WI, and 24 h post-IR Blood and urine: 24 h post-IR	Blood and urine: ??? Tissue: sham-operated mice	Tissue metabolomics: Matrix-assisted laser desorption/ ionization–imaging mass spectrometry (MALDI-IMS) + data calibration by CE-MS. Serum/urine samples: "standard method"
Jouret et al. 2016[29]	Mouse	C57BL/6J mice Male	10 weeks ~20 g	Clamping Clamping of renal pedicles for 30 min.	-	30	Blood (vena cava) Tissue Urine (metabolic cage)	Blood, tissue, and urine: 6 h, 24 h and 48 h post-IR	Blood, tissue and urine: sham-operated mice	Blood/tissue/urine metabolomics: ^{1}H-NMR Serum levels urea & creatinine: COBAS 6000 device (Roche).
Legouis et al. 2020[3]	Mouse	C57Bl/6 J mice Male	10-12 weeks 25–28-g	Clamping Clamping of renal pedicles for 25 min.	-	25	Blood (???) Urine (???)	Blood and urine: 24 and 48 h post-IR Blood (for lactate clearance test): 15, 30, 60, 90, 120 min post-injection of sodium lactate (injection at 6 h post-IR)	Blood and urine: sham-operated mice	Blood lactate and glucose: Aviva Accu-Check glucometer and Novabio StatStrip Xpress lactate meter. SCr: capillary electrophoresis BUN: quantitative colorimetric determination using Stanbio Excel analyser

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Poyan Mehr et al. 2018[30]	Mouse	C57BL/6J mice Male	8-12 weeks ???	Clamping Clamping of renal pedicles for 20 min.	-	25	Blood (???) Tissue Urine (???)	Blood, urine and tissue: 24 h post-IR	Blood, tissue and urine: sham-operated mice	Tissue/urine metabolomics: LC-MS SCr: LC-MS
Rao et al. 2016[31]	Mouse	C57BL/6 mice Male	10-12 weeks ???	Clamping Right nephrectomy, clamping of left renal pedicle for 30 min.	-	30	Tissue	Tissue: 6 and 24 h post-IR	Tissue: sham-operated mice	Tissue lipid concentrations: SWATH-MS Tissue hydroxyoctadeca dienoic acid/ hydroxyeicosatetra enoic acid measurement: LC-MS/MS
Wei et al. 2014[32]	Mouse	C57BL/6 mice Male	9 weeks ???	Clamping Clamping of renal pedicles for 25 min.	-	25	Blood (???) Tissue (cortex and medulla)	Blood and tissue: 2 h, 48 h or 1 week post-IR	Blood and tissue: sham-operated mice	Blood/tissue metabolomics: GC/MS and LC/MS
Zager et al. 2014[33]	Mouse	CD-1 mice Male	??? 30-45 g	Clamping Unilateral clamping of left renal pedicle for 15, 30 or 60 min.	-	15, 30 or 60	Blood (vena cava) Tissue (cortex)	Blood and tissue: after 15, 30, or 60 min WI, and 2 or 18 h post-IR	Blood: sham-operated mice Tissue: contralateral kidney and sham-operated mice	Tissue/blood lactate, pyruvate, glucose and glycogen levels: enzymatic assays
Pig										
Clendenen et al. 2019[34]	Pig	Farm pigs Male	??? 50-55 kg	Clamping Clamping of renal pedicles for 30 min.	-	30	Blood (renal vein)	Blood: pre-IR, after 15 and 30 min WI, and 5 min post-IR	Blood: pre-IR	Blood metabolomics: UHPLC-MS

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Fonouni et al. 2011[35]	Pig	Landrace pigs	??? 26-33 kg	Transplantation Living donor left kidney explantation (30 min), implantation in recipient after 6 h CI. 120 min reperfusion.	6	60 (anastomosis)	Blood (???) Extracellular fluid (microdialysis = MD)	Blood: during procurement, post-I, and 120 min post-IR MD: 10-min intervals during kidney procurement, CI (2 samples in the first and 2 samples at the end of CI), and at 20-min intervals during kidney implantation (WI) and post-reperfusion (120 min).	Blood and extracellular fluid: during explantation procedure	MD: CMA 600 Microdialysis Analyzer Blood analysis: hospital laboratory
Hauet et al. 2000[36]	Pig	Large White pigs Male	??? 41–52 kg	Transplantation Left nephrectomy, kidneys were flushed with EC solution or UW solution. After 48 h CI, heterotopic auto-transplantation was performed and contralateral nephrectomy was carried out.	48	???	Blood (right jugular vein) Urine (metabolic cage)	Blood and urine: two days before kidney preservation (D-2) and at 1, 3, 5, 7, 11, and 14 days (D1–D14) post-IR. Also examined these kidneys 30–40 min after implantation and on the sacrifice day.	Blood and urine: control group uninephrectomized, no flushing or cold preservation	Blood/urine metabolomics: ^{1}H-NMR
Malagrino et al. 2019[37]	Pig	Pigs Female	Juvenile 15-20 kg	Clamping Unilateral clamping of the right renal artery for 120 min.	-	120	Blood (inferior vena cava above the renal veins) Tissue Urine (directly from bladder)	Tissue: 24 h post-IR Blood, urine: pre-IR (before occlusion), after 1 h WI, 0.5 h post-IR, 4 or 6 h post-IR, and 11 h post-IR	Tissue: contralateral kidney Blood and urine: pre-IR samples	Blood/urine metabolomics: ^{1}H-NMR

Article	Species	Breed	Age Weight	Ischemia: transplantation, surgery or clamping	Cold ischemia time (h)	Warm ischemia time (min)	Sample type	Sampling time points	Control(s)	Relevant measurement techniques
Dog										
Maessen et al. 198[93]8	Dog	Mongrel dogs	Adult 18-25 kg	Transplantation Clamping of left renal vessel pedicle for 0 or 30 min. Then kidney explantation and storage on ice. Autologous reimplantation, contralateral nephrectomy.	24 or 48	0 or 30 + ???	Tissue (cortex)	Tissue: after WI, after CI, and 1 h post-IR	Tissue: non-ischemic control	Tissue energy metabolite levels: HPLC
Montanés et al. 1991[39]	Dog	Mongrel dogs	??? 17-34 kg	Clamping Right nephrectomy, clamping of left renal artery for 60 min.	-	60	Blood (femoral artery, left ovarian, or spermatic vein) Tissue (cortex) Urine (left ureter and metabolic cage)	Blood, tissue, and urine: 2 days post-IR	Blood, tissue and urine: sham-operated dogs	Blood/tissue metabolomics: enzymatic assays Scr: enzymatic assay Blood/urine pH and gasses: blood gas analyzer model 168 (Corning Medical)

*For the sake of clarity, the induction of ischemia was classified as "clamping", including several approaches to occlude blood flow, or "transplantation", in which the kidney was removed and transplanted. AV: arterio-venous. BUN: blood urea nitrogen. CE-MS: capillary electrophoresis-mass spectrometry. CE-ToFMS: capillary electrophoresis–time of flight mass spectrometry. CI: cold ischemia. DGF: delayed graft function. fDGF: functional DGF. FIA-MS/MS: flow injection analysis tandem mass spectrometry. GC: gas chromatography. GCxGC-MS: 2-dimensional gas chromatography-mass spectrometry. HPLC-Q-ToF MS: high-performance liquid chromatography–quadrupole-time of flight mass spectrometry. IR: ischemia reperfusion. KIM-1: kidney injury molecule-1. LC: liquid chromatography. MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry. MD: microdialysis. MRI: magnetic resonance imaging. MRS: magnetic resonance spectroscopy. NGAL: neutrophil gelatinase-associated lipocalin. NMR: nuclear magnetic resonance. Post-I: post-ischemia. Pre-I: pre-ischemia. qPCR: quantitative polymerase chain reaction. SCr: serum creatinine. SWATH-MS: sequential window acquisition of theoretical spectra-mass spectrometry. UHPLC: ultra-high performance liquid chromatography. UPLC-MS/MS: ultra-performance liquid chromatography tandem mass spectrometry. WI: warm ischemia. ???: data unknown. *: additionally included study.*

Table 3. Results reported in the publications included by the systematic search.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Rat					
Andrianova et al. 2020[7]	Rat	Serum creatinine (SCr) levels: "After IR, creatinine concentration increased in all animals, varying from 50 to 200–500 μM, indicating that IR caused AKI" *Note: no thresholds given.*	SCr (increase post-IR)	-	Only measured acylcarnitines (ACs) and amino acids (AAs). After IR, we detected 34 metabolites in blood serum, whose levels significantly changed – concentration of 31 acylcarnitines increased, while the content of 3 AAs (tyrosine, tryptophan, and proline) dropped. The most significant changes were observed for malonylcarnitine, which demonstrated a 7-fold increase compared to control, glutarylcarnitine (5-fold increase), decadienoyl.arnitine (4-fold increase), hydroxybutyrylcarnitine (4-fold increase), linoleylcarnitine (4-fold increase), and methylmalonylcarnitine (4-fold increase). Other acylcarnitines showed about a 2-fold increase. The serum levels of the tyrosine, tryptophan, and proline concentration dropped to 60%–70% of their content
Choi et al. 2019[8]	Rat	Not defined	-	-	Principal component analysis: Significant separation following cardiac arrest (CA) and following resuscitation. Citrate, a-ketoglutarate, malate, fumarate, and succinate significantly changed in kidney after cardiopulmonary bypass (CPB) compared with control or CA. Individual metabolites changes: -The only amino acids that were unaffected by CA were glutamine, threonine, alanine, and valine, remaining measured amino acids were significantly decreased. 30 minutes of CPB resuscitation resulted in even more dysregulation; all measured amino acids with the exception of glutamate, alanine, and valine were significantly decreased. -30 minutes of CPB resuscitation resulted in a significant decrease in linoleic and linolenic acids, while stearic acid returned to control levels. The general trend of lipids in the kidney is that they increase after ischemia, but either return to or fall below control levels after resuscitation. -The kidney shows variations in glycolytic and TCA cycle metabolites, such as a significant elevation in 3-phosphoglycerate and oxoglutarate after 20 minutes of CA. However, CPB resuscitation resulted in a significant increase in citrate, oxoglutarate, succinate, fumarate, malate, and oxaloacetate. -The urea cycle metabolites were mostly altered after 30 minutes of CPB resuscitation in the kidney. -Arginine and proline were significantly decreased after CA, but after CPB, arginine, citrulline, ornithine, and proline were all decreased. Changes in acylcarnitine species: CA resulted in elevation in only AC 16:0 when compared with control. Resuscitation resulted in a decrease from CA in AC 2:0, AC 8:0, AC 10:0, AC 14:2, AC 16:1, AC 16:0, AC 18:2, and AC 18:1. AC species that were decreased after resuscitation were AC 6:0, AC 8:0, AC 10:0, AC 14:2, AC 14:1, AC 16:2, AC 16:1, AC 18:2, and AC 18:1. The kidney was unable to normalize ACs after resuscitation, resulting in a generally diminished lipid reserve after 30 minutes of CPB resuscitation. Pathway analysis: In kidney tissue following resuscitation, 46 pathways remained significantly altered compared with control, with nicotinate and nicotinamide metabolism, and phenylalanine metabolism having the lowest q values.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Duran et al. 1990[9]	Rat	Not defined	BUN (no change post-IR)	Kidney weight (increase post-IR)	Only measured AAs: The plasma concentrations of AAs were indistinguishable from control. Cellular AAs post-IR: glutamate, glycine, phenylalanine, and serine in cortical cell water (=derived from tissue) were decreased 3 h after ischemia, but those of the remaining 12 AAs were not different from control. Concentrations of glutamate and glycine had normalized 24 h after blood reflow, leaving only 4 AAs – arginine, phenylalanine, serine and threonine – at decreased concentration.
Gaudio et al. 1991[10]	Rat	EM images of proximal tubule segments: "Following ischemia and 15 min of reperfusion, there were marked cellular alterations typical of ischemic injury".	-	Histology	45 min of ischemia resulted in a significant fall in ATP levels. ATP levels had increased by only a small amount after 2h of reperfusion.
Huang et al. 2018[11]	Rat	Not defined	SCr (increase post-IR) Lactate (tissue and plasma levels increase post-IR)	Histology	Metabolomics: -Palmitate, stearate, linoleate, 1-monopalmitin, 2-monopalmitin, 2-monostearin, and cholesterol appeared to accumulate after 4 h followed by a reduction after 24 h IR. -Reduced glucose levels in both 4 h-IR and 4 h-control (4 h-C) kidneys that were sustained in 24 h-IR as compared to HC (healthy control). Glucose levels were unaltered in plasma of 4 h and 24 h-operated animals. Lactate levels were increased in 24 h-IR as compared to HC, and were also significantly elevated in the plasma of 4 h-operated animals and even to a greater extent after 24 h. -Blood creatinine levels were higher in both 4 h and 24 h post-IR. -Compensatory changes in metabolite levels in the uninjured organ of animals subjected to kidney IR, in particular after 24 h reperfusion: a strong elevation of urea and AMP in contralateral kidneys after 24 h post-IR, which was not observed in the injured kidney counterpart. Adenosine, glutamic acid and glycine levels were increased in a more prominent fashion in contralateral kidneys, particularly after 24 h. Citrate appeared to be elevated in all conditions as compared to control. ATP levels were significantly decreased in 24 h-IR kidney tissue as compared to 24 h-C and HC.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
					Integrated analysis: Hierarchical cluster analysis revealed the existence of five phenotypes: i) Decreased substrates in 4 h-IR and/or 4 h-C compared to HC and 24 h-IR/C. This includes proteins involved in fatty acid (FA) biosynthesis (Acsl6, Acsl4), metabolites involved in energy metabolism (glucose and citric acid); ii) Decreased substrates prevalently in 24 h-IR compared to the other conditions. This group: adenosine, proteins involved oxidation and reduction reactions (Por) and enzyme that play role in the TCA cycle (Pdha1/1). iii) Metabolites increased prevalently in 24 h-C animals as compared to the other conditions. This includes free fatty acids (FFAs) (2-Monostearin, 2-Monopalmitin and linoleate), non-essential amino acids (Glutamic acid, glycine), urea, AMP and creatinine. iv) Proteins and metabolites prevalently increased in 24 h-IR including enzymes involved in oxidative phosphorylation (Ndufa6, Ndufv1, and Ndufs1), fatty acid binding protein (Fabp4), glycolysis enzyme (Hk1) and lactate. v) Enzymes and metabolites elevated in 4 h-IR and 4 h-C such as glucose transporter (Slc5a1), FA transporter (CD36), components of oxidative phosphorylation (ND-1), detoxification enzymes (Adh5, Ugt2b15), mitochondrial biogenesis (Sirt2), FFA metabolism (Cpt1a, Acadsb, Echdc3, palmitate, stearate, 1-monopalmitin) and ketone metabolism (Oxct1).
Lan et al. 2016[12]	Rat	IR injury was monitored by SCr and renal histology. *Note: no thresholds given.*	SCr (no data reported)	Histology	During reperfusion 3, 7, and 14 days after IR, lactate and pyruvate concentrations in cortex and the outer stripe of outer medulla were increased relative to time controls.
Legouis et al. 2020[3]	Rat	Not defined	-	-	Renal gluconeogenesis, selectively quantified by analysis of blood from the renal vein, was reduced in response to IR.
Lindhardt et al. 2020[13]	Rat	Not defined	SCr (no control) BUN (no control) Urinary creatinine (no control)	-	The metabolism measured in vivo by product/pyruvate ratios showed a significant decrease in the ischemic kidney compared with the contralateral kidney when considering all metabolites (lactate, alanine, bicarbonate).

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Liu et al. 2012[14]	Rat	BUN and SCr were two widely used indicators of kidney Injury. There were significant differences in SCr and BUN between the control and IR groups. *Note: no thresholds given.*	SCr (increase post-IR) BUN (increase post-IR)	-	-Most important IR-related metabolites are lysophospholipids and FFAs, including stearoyl-glycerophosphocholine, eicosatrienoyl-glycerophosphocholine, oleoyl-glycerophosphocholine, palmitoyl-glycerophosphocholine, linoleoyl-glycerophosphocholine, linolenoyl lysolecithin, stearic acid, oleic acid, linoleic acid, arachidonic acid and eicosapentaenoic acid. -Nitrotyrosine (oxidative product of tyrosine) significantly increased in the IR group. -Increased hydroxymethylphenidate after IR. -Carnitine and acetyl-carnitine decreased during IR. -Saturated fatty acids and unsaturated fatty acids displayed different changes in the IR group. However, stearic acid made more contribution than polyunsaturated fatty acids (PUFAs) to discriminate between the IR and control.
Nielsen et al. 2017a[15]	Rat	Functional kidney parameters showed consistent signs of renal IR injury with an elevated plasma creatinine level of 91% and a reduced creatinine clearance and BUN level of 44% and 30%, respectively, when comparing pre-surgery with post-surgery values. *Note: no thresholds given.*	SCr (increase post-IR) BUN (increase post-IR) Creatine clearance (decrease post-IR) Urine output (non-significant increase post-IR)	Histology Body weight (non-significant decrease post-IR) Kidney weight (increase post-IR)	An elevated malate/fumarate ratio of 339% in the ischemic kidneys compared to that in the contralateral kidney was found.
Nielsen et al. 2017b[16]	Rat	We demonstrated that increased BUN and plasma creatinine occurred in both unilateral IR groups compared with sham-operated rats. Together, this indicates that renal IR resulted in acute renal insufficiency. *Note: no thresholds given.*	SCr (increase post-IR) BUN (increase post-IR)	Renal KIM-1 and NGAL mRNA expression (increase post-IR) Body weight (decrease post-IR) Kidney weight (increase post-IR (60 min ischemia))	Significant decrease of 18–25% in the pyruvate-to-lactate conversion in the 60-min postischemic kidney compared with the contralateral kidneys and kidneys from sham-operated rats. No reduction in pyruvate-lactate turnover was observed in the 30 min IR Group. The alanine-to-pyruvate and bicarbonate-to-pyruvate ratio similarly showed a decrease of 44% and 59%, respectively, in the postischemic kidney, no reduction in metabolite turnover was seen in the 30 min IR Group. The lactate-to-bicarbonate ratio was significantly shifted toward anaerobic glycolysis in the 60-min postischemic kidney by 44%. No statistical difference was found between alanine metabolism and aerobic glycolysis (alanine-to-bicarbonate ratio), but the lactate-to-alanine ratio was significantly increased by 25%, and a small increase in lactate-to-alanine ratio of 23% was also seen in the 30-min IR group. Pyruvate-to-total carbon signal and a total carbon kidney fraction were calculated for each kidney of the sham-operated and unilateral IR rats, which yielded a significantly elevated ratio of 6% for the 60-min postischemic kidney. Increase of lactate in the 60-min IR group of 178%, and no significant increase in the 30-min group.

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Nielsen et al. 2020[17]	Rat	Not defined	SCr (increase post-IR for longer ischemia, stable levels post-IR for short ischemia, (partially) recover 7 days post-IR)	-	Acute alterations in the ischemic re-perfused kidneys overall metabolic phenotype were seen between the ischemic/early perfusion stage (2 min) and after 1 hour of perfusion, showing a compensatory mechanism in the contralateral kidney 60 min after reperfusion. The acute change in the lactate-to-bicarbonate ratio 60 min after reperfusion was not correlated with the early signature 2 min after reperfusion. The acute metabolic reprogramming seen at 60 min was driven by post release compensation in the contralateral kidney as well as downregulation of the lactate-to-bicarbonate balance in the ischemic kidney. The in vivo response that was seen 24 hours and 7 days following ischemic injury showed a similar tendency towards a general reduction of the overall metabolism in the ischemic kidney as well as a compensatory increased anaerobic metabolism shown by increased lactate production when compared to the aerobic metabolism, shown by CO2 and HCO3-production. Both 20 min and 40 min ischemia in the kidney results in a tendency towards a metabolic reprogramming from 24 hours to 7 days, with a statistically significant shift observed in the 40 min group. The metabolic phenotype at 24 hours, with reduced lactate-to-bicarbonate ratio, is positively correlated with the lactate-to-bicarbonate ratio at 7 days. A positive correlation was found in the lactate-to-bicarbonate ratio between 24 hours and 7 days. While no such correlation was found between the perfusion stage (2 min) and 60 min. By looking at the 20 min and 40 min group, one group with a large variance covering the 30 min acute insult, we compared the overall metabolic pattern from the initial 2 min – 7 days between the ischemic and contralateral kidney. This combination shows a significant change already at 60 min which persists throughout the 7 days. These findings are supported by a tendency towards a correlation between the lactate-to-bicarbonate ratio.
Peto et al. 2018[18]	Rat	Not defined	Lactate (blood lactate increase post-IR, pH decrease)	-	-Blood lactate concentration in parallel to pH increased by the end of the observed reperfusion period in all groups. In Control group the change was not significant, but in IR, where the largest rise was found, it was. -No significant change in glucose concentration.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Serkova et al. 2005[19]	Rat	Graft function: clinical appearance, histology, SCr, and urine output. *Note: no thresholds given.*	SCr (increase post-IR) Anuria	Histology Clinical appearance (behavior, fur, etc.) Body weight (no change post-IR)	Tissue: -Cold storage in UW solution: significant increase in glycogen and other carbohydrates was observed at 24 and 42 hours of CI. The lactate concentration was greatly increased, up to 380% during CI. In the lipid extracts, a decrease of PUFAs was seen in CI groups versus native kidney. There were no significant differences in metabolic composition at the end of 24 and 42 hours of CIT. -Transplanted kidneys exposed to reperfusion for 24 hours demonstrated characteristic changes: most pronounced difference between the post-IR and post-I groups was the dramatic increase of allantoin. Allantoin concentrations were low in the native kidney and at the end of CI, but increased significantly after 24 h reperfusion. Stepwise logistic regression analysis revealed that from 30 metabolites quantified from kidney extracts, only two–allantoin and PUFA – were different among study groups. Blood: -In 6/8 animals in the native group, allantoin concentrations were below the limit of quantification for NMR. - Allantoin peaks appeared in the blood in both CI groups following reperfusion. Allantoin concentrations were higher in transplanted rats with 42-hour cold storage when compared with 24-hour cold storage. -Trimethylamine-oxide (TMAO) correlated well with CIT. TMAO concentration correlated well with elevated allantoin levels. -Uric acid concentrations were below the linear range for the assay.
Shen et al. 2017[20]	Rat	Renal injury confirmed by histology: disrupted kidney structure.	-	Histology	-All metabolites enriched in the biosynthesis of unsaturated fatty acids were augmented in IR group in contrast to the control group. -D-glucose, lactic acid and cholesterol were differentiated significantly between control and IR group.
Tani et al. 2019[21]	Rat	Not defined	Lactate (tissue levels increase post-IR) SCr (increase post-IR) BUN (increase post-IR)	-	-Energy charge decreased upon ischemia, and increased upon reperfusion. -Total adenine nucleotide (TAN), ATP and ADP levels are decreased upon ischemia, and increased only slightly upon reperfusion. AMP showed hardly any difference between stationary state and ischemia, but decreased during reperfusion to a lower level. -Hypoxanthine, xanthine, and uric acid levels are high after ischemia, but return to baseline after reperfusion. -All groups in the ischemic state showed an increase in IMP level and a decrease in deoxyadenosine triphosphate (dATP) level, and a decrease in the adenine level after reperfusion. -TAN' (the sum of all detectable purine metabolites excluding ATP, ADP, and AMP, thus including dATP, PRPP, adenosine, adenine, inosine, IMP, hypoxanthine, xanthine, and uric acid) increased during ischemia and dropped to approximately baseline after reperfusion again. -Drastic metabolic changes by the IR procedure: triphosphate compounds in purine/pyrimidine metabolism pathways, nicotinamide adenine dinucleotide (NAD+), uridine diphosphate (UDP)-glucose, kynurenine, citrulline and amino acids such as ornithine, isoleucine, leucine, and tryptophan. -Marked accumulation of hydrolysis products, such as lactate and ß-hydroxybutyrate.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Trifillis et al. 1984[22]	Rat	SCr and BUN were used as indices of renal function. *Note: no thresholds given.*	SCr (increase post-IR) BUN (increase post-IR)	Survival	<u>1 h ischemia and variable reperfusion times:</u> ATP levels after 1 h of clamping decreased significantly to 18% of control levels. Upon release of the clamp, ATP levels began to increase after 0.25 h of reflow. ATP concentrations finally returned to control levels after 24 h of reflow. ADP levels remained relatively unchanged. AMP levels doubled after 1 h of ischemia but promptly returned to control levels after 0.25 h of reflow. Therefore, changes in AXP levels paralleled those of ATP levels, i.e., AXP levels were not fully restored to control levels until 24 h of reflow. The energy charge decreased to 50% of the control value after 1 h of ischemia but returned to control levels after 6 h of reflow. Lactate levels reached 13-fold control levels after 1 h of clamping and remained significantly elevated until 24 h of reflow when they returned to control levels. <u>Variable ischemia times and 24 h of reperfusion:</u> 120 min of ischemia resulted in death within the 24-h reflow period. SCr levels increased significantly after 60 and 90 min of ischemia followed by 24 h of reflow. In the left kidney, adenine nucleotides, lactate, and inorganic orthophosphate levels were restored essentially to control levels after 30 min of ischemia followed by 24 h of reflow. Adenine nucleotide levels were partially restored but remained significantly lower than control levels after 60 and 90 min of ischemia and 24 h of reflow. Lactate levels were restored to controls after 15-90 min of ischemia followed by 24 h of reflow. Inorganic orthophosphate and the phosphorylation state were significantly different from controls only after 60 min of ischemia followed by 24 h of reflow. Adenine nucleotide, lactate, and inorganic orthophosphate content of the right kidney were not significantly different from that of the left kidney at any time period studied, i.e., the time course and magnitude of metabolite restoration following 24 h of reflow was the same in both kidneys.
Varga et al. 2019[23]	Rat	Not defined	Lactate (serum levels increase post-IR, pH decrease) SCr (increase post-IR)	-	Lactate and potassium concentration significantly increased in IR (measurements after 120 min). IR decreased the pH.
Mouse					
Beier et al. 2020[24]	Mouse	Not defined	-	-	We found 30 metabolites elevated in IR. Among the uniquely increased metabolites in IR (compared to acute cellular rejection), the highest fold difference was observed for the lysine catabolite saccharopine. Also the downstream products 2-aminoadipate and glutarate, but not the parent substrates lysine and α-ketoglutarate, were increased in IR.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Chihanga et al. 2018[25]	Mouse	SCr, urinary NGAL, urinary NGAL/ urinary creatinine ratios, and urinary creatinine levels confirmed AKI 24 h post-IR. *Note: no thresholds given.*	SCr (increase post-IR) Urinary creatinine (decrease post-IR)	Histology Urinary NGAL (increase post-IR) Urinary NGAL/urinary creatinine (increase post-IR)	-Urinary concentrations of many metabolites before IR changed dramatically after IR. Cis-aconitate, citrate, creatine, phosphocreatine, putrescine, sarcosine, succinate, taurine, n-nitrosodimethylamine, trimethylamine, uracil, and trimethylamine N-oxide, galactaric acid, guanine and hippurate all decreased following IR. Nicotinamide-n-oxide, trigonelline, 2-oxoglutarate, and 2-oxoisocaproate were absent in urine following injury. Glucose, lactate, alanine, valine, and leucine had higher urine concentrations following IR. -NMR spectroscopy of plasma collected 24 h post-IR indicated no new metabolites post-IR except for creatinine. Metabolic profiling of kidney tissue extracts indicated no new metabolites following IR.
Cho et al. 2017[26]	Mouse	There was a significant increase in acute tubular injury in the IR group compared to the sham group.	-	Histology	-The levels of adenosine and 5'-deoxy-5'-methylthioadenosine were higher in IR-injured kidney. IR-injured kidney was characterized by decreased phosphatidylethanolamine (PE) 20:3/20:4, and betaine aldehyde levels in kidney samples, as well as increased cell membrane constituents. -Increased serum levels of fatty acids; in particular, 4,14-dimethyl-hexadecanoic acid, 15-eicosenoic acid, 3,5-dimethyl-tetradecanoic acid, cis-8,11,14-eicosatrienoic acid, and 3-oxo-2-pentyl-cyclopentaneoctanoic acid. The acyl-carnitines 2-octenoylcarnitine and 2-hydroxylauroyl-carnitine were decreased in the urine and serum, respectively. Levels of arachidonic acid and cis-4,7,10,13,16,19-docosahexaenoic were lower following IR than in the sham group.
*Chouchani et al. 2014[27]	Mouse	Not defined	-	-	Increase of hypoxanthine and xanthine after ischemia. Succinate accumulation during ischemia, recovery to baseline after 5 min of reperfusion.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Fujii et al. 2019[28]	Mouse	Transient ischemia for 10 min was sufficient to cause significant renal injury with increased NAG in urine and decreased creatinine clearance. No histological damage. *Note: no thresholds given.*	Creatinine clearance (decrease post-IR)	Histology (note: no changes) Urinary NAG (increase post-IR)	-In the normal kidney sections, high-energy adenine nucleotides (ATP and ADP) were significantly rich (vs inner medulla) in both the cortex and the outer medulla (outer stripe of outer medulla (OSOM) and inner stripe of outer medulla (ISOM)). -ATP and total adenylates in the cortex and OSOM decreased by transient ischemia for 10 minutes: -ATP in the cortex and the outer medulla decreased within a minute after the clipping procedure, and adenosine, inosine, and hypoxanthine increased in every region of the kidney. In particular, the content of ATP decreased by 45% within a minute of ischemia, and the decrease reached 84% during 10 min ischemia. In the inner medulla, ATP did not decrease within a minute, and significant decline first became evident at 10 min after clipping. AMP increased in every region of the kidney during the ischemia. Energy charge value decreased in the whole kidney within a minute. Accumulation of adenosine in the OSOM disappeared after the clipping procedure, which was associated with the increase of adenosine in regions of the kidney except for the OSOM. -Inosine and hypoxanthine increased in every region of the kidney within a minute and in parallel to AMP, and the degrading changes of adenylates progressed during 10 min ischemia. Metabolome analysis revealed increase of xanthine and uric acid in the ischemic kidney. -In the reperfusion sections, restoration of ATP in the renal cortex and OSOM was not complete, and ATP showed a 24% decrease when compared with sham sections. In contrast, the restoration of ATP in the ISOM and inner medulla was sufficient. Total adenylates in the cortex and OSOM decreased after 24 h reperfusion, total adenylates were maintained in the ISOM and inner medulla: total adenylates and ATP in the cortex and OSOM after 24 h reperfusion demonstrated prolonged loss. The breakdown products of ATP were increased in the whole kidney by 10 min ischemia, almost recovered to the original content after 24 h reperfusion. A prolonged loss of ATP was significant after 10 min ischemia. -NADH showed a 5-fold increase in all regions of the kidney subjected to 10 min ischemia. The increase in NADH in the cortex and OSOM persisted during 24 h of reperfusion, whereas it significantly decreased (vs 10 min ischemia) in the ISOM and inner medulla.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Jouret et al. 2016[29]	Mouse	The renal function was monitored by SCr and serum urea levels. IR-exposed mice showed a significant increase of both AKI parameters. *Note: no thresholds given.*	SCr (increase post-IR, recover 48 h post-IR) Serum urea (increase post-IR, recover 48 h post-IR) Lactate (urinary and tissue levels increase post-IR)	-	Urine: -Urine levels of taurine, lactate and glucose were steadily increased after IR, urine levels of trimethylamine were significantly reduced. -Pathways significantly affected by renal IR: gluconeogenesis and taurine/hypotaurine metabolism at 6 and 24h reperfusion. Protein biosynthesis, glycolysis and galactose and arginine metabolisms appeared essential at 48h reperfusion. Allantoin increased 24 h post-IR, but decreased 48 h after. Tissue: -Similar discriminations in tissue: changes in levels of lactate, fatty acids, choline and taurine. -The identification of metabolites, whose increased abundance reached significance in loading plots included fatty acids (and modified lipoproteins), lactate and N-acetyl groups of glycoproteins. -Levels of taurine and myo-inositol were decreased in kidneys from IR mice in comparison to sham animals. -Analysis of metabolites at 6h and 24h reperfusion: taurine/hypotaurine and betaine metabolisms were significantly affected by renal IR. -At 48h post reperfusion, IR-associated cascades were protein biosynthesis, biotin and taurine/hypotaurine metabolism. Serum: Serum analysis could not discriminate sham-operated from IR-exposed animals.
Legouis et al. 2020[3]	Mouse	Not defined	SCr (increase post-IR) BUN (increase post-IR)	-	Mice exposed to severe IR injury displayed increased urea and creatinine levels, together with a decrease in glucose and increase in lactate serum levels at 48 h, whereas urinary glucose and lactate were unchanged except for one outlier. Blood lactate clearance was impaired in the IR group after intraperitoneal injection of sodium lactate. Increase in blood glucose following lactate injection was reduced in the IR group.
Poyan Mehr et al. 2018[30]	Mouse	SCr was measured as a measure for renal function and postischemic injury. *Note: no thresholds given.*	SCr (increase post-IR)	-	204 metabolites measured, 27 were more than twofold increased in postischemic urines compared to controls including several sugars and amino acids, a pattern consistent with tubular impairment. Among these metabolites was quinolinate, an intermediate in the de novo NAD+ biosynthetic pathway from tryptophan. Many other metabolites, including amino acids and acyl-carnitines, were differentially regulated in urine of AKI mice.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Rao et al. 2016[31]	Mouse	Renal injury confirmed by SCr levels and histology. *Note: no thresholds given.*	SCr (increase post-IR)	Histology	Only measured lipids: -Four lipids were changed (all increases) to a statistically significant extent at 6 h after IR. Of these, three were identified as ether-linked phospholipids (one an abundant phosphatidylcholine (PC): PC O-38:1, and two PEs: an abundant PE O-42:3 and a minor PE O-40:4). The two abundant ether lipids were: PC O-38:1 (PC O-18:0, 20:1) and PE O-42:3 (PE O-20:1, 22:2). PC O-38:1 is a plasmanylcholine, while PE O-42:3 is a plasmalogen. -Many more lipids were changed to a statistically significant extent at 24 h after IR. The abundant PC O-38:1 remained elevated in IR kidneys at 24 h compared with the 24-h sham group, the low-abundance PE O-40:4 was present at comparably low levels in kidneys of both IR and sham mice at 24 h. PE O-42:3, was present at high levels but was decreased at 24 h compared with its sham control group, this lipid was increased relative to its sham control group at 6 h post-IR. All ether-linked PEs and PEs detected 24 h post-IR were reduced with AKI. -No statistically significant differences in major hydroxyoctadeca dienoic acids and hydroxyeicosatetra enoic acids or linoleic and arachidonic acids were detected in kidneys of sham and IR animals at 6 h post-IR.
Wei et al. 2014[32]	Mouse	Renal function: statistically significant differences in SCr or BUN levels compared to sham condition. *Note: no thresholds given.*	SCr (increase post-IR, peak 48 h post-IR, recover 1 week post-IR) BUN (increase post-IR, peak 48 h post-IR, recover 1 week post-IR) Lactate (tissue and serum levels decline post-IR, but recover)	-	-The changes started in renal cortex, followed by medulla and plasma. -Increased allantoin levels (specifically cortex, not medulla). -Elevated serum β-hydroxybutyrate levels. -The kidney cortex and the plasma samples showed early decreases in glucose and lactate, but recovery to near-sham levels by 1 week reperfusion time. -Some metabolites, such as 3-indoxyl sulfate, were induced at the earliest time point of renal IR. -There was a notable switch of energy source from glucose to lipids. -Decreased polyols for osmotic regulation. -Several pathways involved in inflammation regulation were induced. -Late induction of prostaglandins.
Zager et al. 2014[33]	Mouse	Severity of AKI was assessed by BUN and plasma creatinine concentrations and renal cortical NGAL mRNA levels. *Note: no thresholds given*	SCr (increase post-IR) BUN (increase post-IR)	-	Ischemia induced persistent pyruvate depletion. During ischemia, decreasing pyruvate levels correlated with increasing lactate levels. During early reperfusion, pyruvate levels remained depressed, but lactate levels fell below control levels. During late reperfusion, pyruvate depletion corresponded with increased gluconeogenesis (pyruvate consumption).

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Pig					
Clendenen et al. 2019[34]	Pig	Not defined	Lactate (serum levels increase post-IR) SCr (increase post-IR)	-	-Lactate increased in response to IR. Glutamate accumulation with a serum increase of 2.7 times from baseline occurring exclusively after reperfusion. Hypoxanthine increased 4 times baseline. IR changed arginine, proline, creatine and polyamine metabolism. Arginine decreased. Proline increased 1.1 times baseline following IR. Creatinine with increased 1.6 times baseline. Arginine consumption and accumulation of ornithine, polyamines (putrescine, spermidine and spermine) were observed upon IR. Spermine increased 5 times baseline levels, with spermidine following a similar trend. Putrescine increased. -Small molecule metabolites involved in redox homeostasis (e.g. reduced glutathione–GSH, cysteine, carnosine, kynurenine, taurine and hypotaurine) and, in general, metabolites involved in glutathione turnover (5-oxoproline) or sulphur metabolism (taurine, hypotaurine, methionine, GSH) were affected. Taurine increased upon reperfusion. No substantial increases in the post-IR circulating levels of carnitine, tryptophan and serotonin.
Fonouni et al. 2011[35]	Pig	Not defined	SCr (no "substantial" differences) BUN (no "substantial" differences) Lactate (extracellular fluid levels peak post-IR, but recover to baseline)	-	Baseline (BL) value = measured parameters in donors at the beginning of the graft procurement. Baseline: Glucose 0.56 mM, lactate 0.46 mM, pyruvate 12.17 μM, glutamate 19.75 mM, glycerol 19.58 μM Procurement: Glucose increase to 1.11 mM, lactate increase 0.54 mM, pyruvate increase 28.03 μM then decrease, glutamate increase to ~40 mM, glycerol similar to BL CIT: Glucose decrease to 0.23 mM, lactate decrease until halfway then increase to 0.35 mM, pyruvate short increase to 20.02 μM then decrease to 4.85 μM, glutamate increase to 82.60 mM, glycerol increase at end to 54.76 μM WIT: Glucose decrease 40 min to 0.14 mM then sharp increase to 0.48 mM, lactate increase to 0.75 mM, pyruvate increase to 10.18 μM, glutamate increase to 131 mM, glycerol increase to 118.22 μM Reperfusion: Glucose increase 40 min 1.47 mM then decrease to 0.73 mM, lactate increase 20 min to 1.07 mM then decrease to 0.58 mM, pyruvate increase 40 min to 29.97 μM then decrease to 17.80 μM, glutamate increase 20 min to 161.60 mM then decrease 40 min 41.03 mM then steady, glycerol increase 40 min to 236.70 μM then decrease to 19.60 μM

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Hauet et al. 2000[36]	Pig	Death: acute renal failure confirmed by histological analysis. These results were associated with prolonged oliguria or anuria. Assessment of renal function: creatinine clearance and fractional excretion of Na+ demonstrated reduced renal function. *Note: no thresholds given.*	Creatinine clearance (decrease post-IR, recover slightly)	Survival Urinary NAG excretion (increase post-IR, recover slightly) Fractional Na^+ excretion (increase post-IR, recover to near-baseline)	-The urinary lactate/Cr ratio was significantly greater in the IR groups vs control. -Urinary citrate/Cr level was higher in control vs ischemia.
Malagrino et al. 2019[37]	Pig	The animals showed changes characteristic of AKI: increased SCr, serum NGAL, fractional excretion of sodium, potassium and chloride and increased glucose and protein in urine. The most important result for the diagnosis to AKI was based on histological analysis, which showed acute tubular necrosis. An increase of nitrated protein in serum and urine was also observed. *Note: no thresholds given.*	SCr (increase post-IR)	Histology Serum NGAL (increase post-IR) Fractional Na^+, K^+ and Cl^- excretion (increase post-IR) Urinary glucose (increase post-IR) Urinary protein (increase post-IR)	Serum: -Metabolites that showed a quick increase or decrease after 60 min of ischemia, followed by a progressive return to baseline after reperfusion: L-glutamate, L-serine, N-isovaleroylglycine, L-methionine, L-proline, 2-aminobutyrate, and choline. These metabolites discriminate between the pre-ischemic and ischemia periods, as well as between the ischemic and 11 h post-reperfusion periods. However, as they returned to basal levels, they do not discriminate between the pre-ischemia and reperfusion periods Urine: Focus on urinary metabolites increased immediately after the reperfusion (0.5 h post-reperfusion), the result of "washing" the ischemic kidney. A sharp increase or decrease in 0.5 h post-reperfusion period compared with the pre-ischemia period: 3-hydroxybutyrate, 3-hydroxyisovalerate, methylguanidine, 3-aminoisobutyrate, trigonelline, betaine, glycerol, trimethylamine, carnosine, citrate, N-phenylacethylglycine, pyruvate and 1-methylnicotinamide. These metabolites were able to discriminate between the 0.5 h post-reperfusion and pre-ischemia periods. Pathway and network analysis: The metabolites identified were overrepresented in the canonical pathways of amino acids degradation, lipid metabolism, molecular transport, small molecule biochemistry, cell cycle, cellular assembly and organization.

Article	Species	Injury definition	Renal function clinical markers	Additional damage markers	Results on metabolome
Dog					
Maessen et al. 1989[38]	Dog	Post-transplant viability	-	Survival	Energy metabolites measured: ATP, ADP, AMP, GTP, GDP, GMP, IMP. CI with/without WI: -24 h CI: adenine nucleotide and guanine nucleotide contents decreased 30%. IMP increased. -48 h CI: no differences with 24 h CI. -30 min WI prior to CI: adenine and guanine nucleotide content already decreased before start of CI, increase of IMP. CI of 24 h after 30 min of WI did not affect content of adenine and guanine nucleotides, but IMP levels further increased. Prolonged CI to 48 h further decreased adenine and guanine nucleotide levels, while IMP did not show an additional increase. Reperfusion: 1h of reperfusion after 24 or 48 h of CI resulted in increase in guanine nucleotide pool. The adenine nucleotides remained unaffected in the 24 h group, but dropped in the 48 h group. IMP levels were greatly reduced in both. When 30 min WI was applied prior to CI, some different patterns were observed. In WI-groups, adenine nucleotide levels dropped in 24 h groups but remained unaffected in 48 h groups after 1 h reperfusion. The effect on guanine nucleotide and IMP levels did not differ from non-WI groups. Following reperfusion, non-WI groups showed higher (ATP+ADP)/AMP ratios.
Montanés et al. 1991[39]	Dog	Not defined	Creatinine clearance (decrease post-IR) BUN (increase post-IR) Lactate (systemic serum levels similar post-IR, kidney-specific clearance decreased post-IR)	-	Systemic blood: no significant changes in pH, bicarbonate, glutamine, glutamate, alanine, lactate, and pyruvate. Kidney specific (AV sampling): no significant changes in slutatmine, glutamate, alanine, and pyruvate. Decreased lactate clearance. Renal cortex: decreased glutamine, glutamate, ADP, AMP, and TAN. No significant changes in α-ketoglutarate, aspartate, lactate, pyruvate, alanine, and ATP. Urine: increased fractional excretion of lactate and pyruvate.

*Results are (partially) quoted or paraphrased from the texts. AA: amino acid. AC: acylcarnitine. AV: arterio-venous. BUN: blood urea nitrogen. CA: cardiac arrest. CPB: cardiopulmonary bypass. CI: cold ischemia. CIT: cold ischemia time. dATP: deoxyadenosine triphosphate. DGF: delayed graft function. FA: fatty acid. fDGF: functional DGF. FFA: free fatty acid. IR: ischemia reperfusion. ISOM: inner stripe of outer medulla. KIM-1: kidney injury molecule-1. NAG: N-acetyl-β-D-glucosaminidase. NGAL: neutrophil gelatinase-associated lipocalin. OSOM: outer stripe of outer medulla. PC: phosphatidylcholine. PE: phosphatidylethanolamine. Post-I: post-ischemia. Pre-I: pre-ischemia. PUFA: poly unsaturated fatty acids. SCr: serum creatinine. TMAO: trimethylamine-oxide. WI: warm ischemia. WIT: warm ischemia time. *: additionally included study.*

Table 4. An overview of the most commonly used definitions of delayed graft function (DGF) and acute kidney injury (AKI) in clinical practice, based on Mallon et al.[40] and Shin et al.[42] (GFR: glomerular filtration rate. RRT: renal replacement therapy.)

Delayed graft function[40]	
Most commonly used	Requirement for dialysis in the first postoperative week
Dialysis-based	Requirement for dialysis in the first postoperative week excluding the first 24 h
	Requirement for two or more episodes of dialysis in the first postoperative week
	Requirement for dialysis in the first 10 days postoperatively
Functional (creatinine-based)	Failure of a fall in serum creatinine of 10% on 3 consecutive days in the first postoperative week
	Serum creatinine at postoperative day 7 >2.5 mg/dL (=221 μM)
	Serum creatinine at postoperative day 10 >2.5 mg/dL (=221 μM)
	Fall in ratio of serum creatinine of postoperative days 1 and 2 of at least 30%
Combination	Dialysis in first week or failure of serum creatinine to fall in first 24 h
	Dialysis in the first week or serum creatinine at postoperative day 7 >2.5 mg/dL (=221 μM)

Acute kidney injury[42]						
RIFLE classification		*AKIN classification*		*KDIGO classification*		*All*
	Serum creatinine		*Serum creatinine*		*Serum creatinine*	*Urine output*
Risk	≥1.5 times baseline, or ≥25% decrease in GFR	Stage 1	≥0.3 mg/dl (=56.52 μM) increase, or ≥1.5 times baseline within 48 h	Stage 1	≥1.5-1.9 times baseline within 7 days, or ≥0.3 mg/dL (=56.52 μM) increase within 48 h	<0.5 mL/kg/h for >6 h
Injury	≥2 times baseline, or ≥50% decrease in GFR	Stage 2	≥2 times baseline	Stage 2	≥2.0-2.9 times baseline within 7 days	<0.5 mL/kg/h for 12 h
Failure	≥3 times baseline, or increase to ≥4 mg/dL, or ≥75% decrease in GFR	Stage 3	≥3 times baseline, or increase to ≥4.0 mg/dL (=353.6 μM) with acute increase of >0.5 mg/dL (=44.2 μM), or initiation of RRT	Stage 3	≥3 times baseline within 7 days, or increase to ≥4.0 mg/dL (=353.6 μM) with acute increase of >0.5 mg/dL (=44.2 μM), or initiation of RRT	<0.3 mL/kg/h for 24 h, or anuria for >12 h

due to their limited follow-up time (Table 2/3). In fact, most preclinical studies (24/35) relied on follow-up times of 24 hours or less, and in 11 studies the follow-up time was even limited to 120 minutes or less. Only 10/35 studies reported a follow-up of more than 24 hours, nine of which addressed aspects of recovery, including one study that exclusively relied on histology as an end-point. For one study, post-reperfusion follow-up time was not reported.

I/R injury was induced by unilateral clamping with the functional, contralateral kidney left in place in 14/35 studies, while 11/35 applied bilateral clamping, 5/35 performed unilateral nephrectomy prior to IR, and one induced transient cardiac arrest. A minority of studies (4/35) applied kidney transplantation as a model of I/R injury. Autotransplantation and contralateral nephrectomy following reimplantation were performed in two studies. One study transplanted an allograft in a bilaterally nephrectomized recipient, and one study did not report whether nephrectomy was performed.

Biomaterial sampling

Diverse sampling protocols (both with regard to the type of biomaterial collected and the timing of the sampling) are applied among the studies (Table 2). The majority of studies (24/27) that include blood sampling, relied on peripheral blood samples (9/24 studies did not specify sampling location). Organ-specific measurements using renal vein blood was performed in 3/27 studies: in rats, pigs and dogs. One of the peripheral blood sampling studies performed kidney-specific sampling through microdialysis. Single post-reperfusion sampling was reported in 13/27 studies. All other studies concerned multiple sampling (two or more consecutive post-reperfusion blood samples).

Eleven studies included urine samples. Urinary sampling was generally (6/11) achieved by means of metabolic cages, and consequently cumulative samples were reported. One porcine and dog study sampled directly from the bladder or ureter, respectively. Three studies did not specify the means of urine collection.

Most studies (17/28) that included tissue-based analysis for metabolomic profiling or as a read-out of injury were based on a single sampling point, both for smaller and larger animals. Studies reporting serial timepoints all relied on biopsies from separate animals. The majority of the studies (18/28) used whole organs, 10/28 (including two studies that also applied *in vivo* magnetic resonance imaging (MRI)) performed a region-specific analysis (i.e. cortex and medulla).

A large variety of *ex vivo* and *in vivo* analysis platforms was used among the studies. *Ex vivo* analysis for metabolic strategies was diverse and included mass spectrometry and/or nuclear magnetic resonance (NMR)-based platforms, as well as more traditional biochemical assays (gas/liquid chromatography and/or enzymatic techniques). *In vivo* measurements were performed in 4/35 studies (all rat), and concerned ^{1}H-MRI combined with hyperpolarized ^{13}C-MRI.

An extensive metabolomic profile was included in 16/35 studies. Other studies applied a focused (targeted) approach, and reported (sub-)aspects of the metabolome, e.g. exclusively amino acids, lipids or high energy phosphates. For the sake of clarity, extracted data was clustered along the lines of metabolic competence (energy (high energy phosphate) and/or redox status), and the primary metabolic routes (glycolysis,

tricarboxylic acid (TCA) cycle, fatty acid oxidation (β-oxidation), amino acids) to allow for comparison of metabolic signatures.

Metabolic outcomes

Metabolic aspects (i.e. reported contrasts between cases and controls) from each experimental study are summarized in a qualitative overview (Figure 2; quantitative data was categorized to minimize interference caused by differences in measurement techniques and normal values). Data regarding metabolic recovery was only available for studies reporting successive timepoints (Table 2).

Reported metabolic profiles for experimental I/R injury are diffuse, and often incomplete (Figure 2). For example, the metabolic clues required to assess post-reperfusion metabolic (in)competence (i.e. information on energy equivalents) were only available for a subset (10/35) of studies. Based on these studies, most (6/7) rodent studies and the dog studies indicate post-reperfusion metabolic competence (i.e. recovery of high energy phosphates and/or absent release of products indicating ATP/GTP degeneration). Elevated levels of high energy phosphate breakdown products were reported in one pig study (5-minutes post-reperfusion), but no information was available for later timepoints; it is therefore unclear whether this reflects post-reperfusion wash-out or I/R injury.[4] Reported aspects of the post-reperfusion redox status (tissue acetoacetate/β-hydroxybutyrate, or lactate/pyruvate ratio (plasma, serum, dialysate)) and their dynamics following experimental IR vary substantially.

Tissue and blood glucose levels are generally reported to be decreased or recovering after experimental IR (Figure 2). Blood lactate levels are mostly increased following IR, while its tissue contents are reported variably. Among rodent studies, remarkable variations were observed in the dynamics of TCA cycle intermediates.[8,20] The limited data for porcine studies is relatively consistent, generally showing increased circulating levels of TCA intermediates following IR.

Aspects of β-oxidation were only reported in a minority (n=10) of rodent studies, and conclusions were variable (Figure 2). Similarly, reported aspects of amino acid metabolism and intermediates are limited and conclusions are variable: tissue levels are decreased or stable following IR in rats and dogs, whilst levels in murine tissues are highly variable.

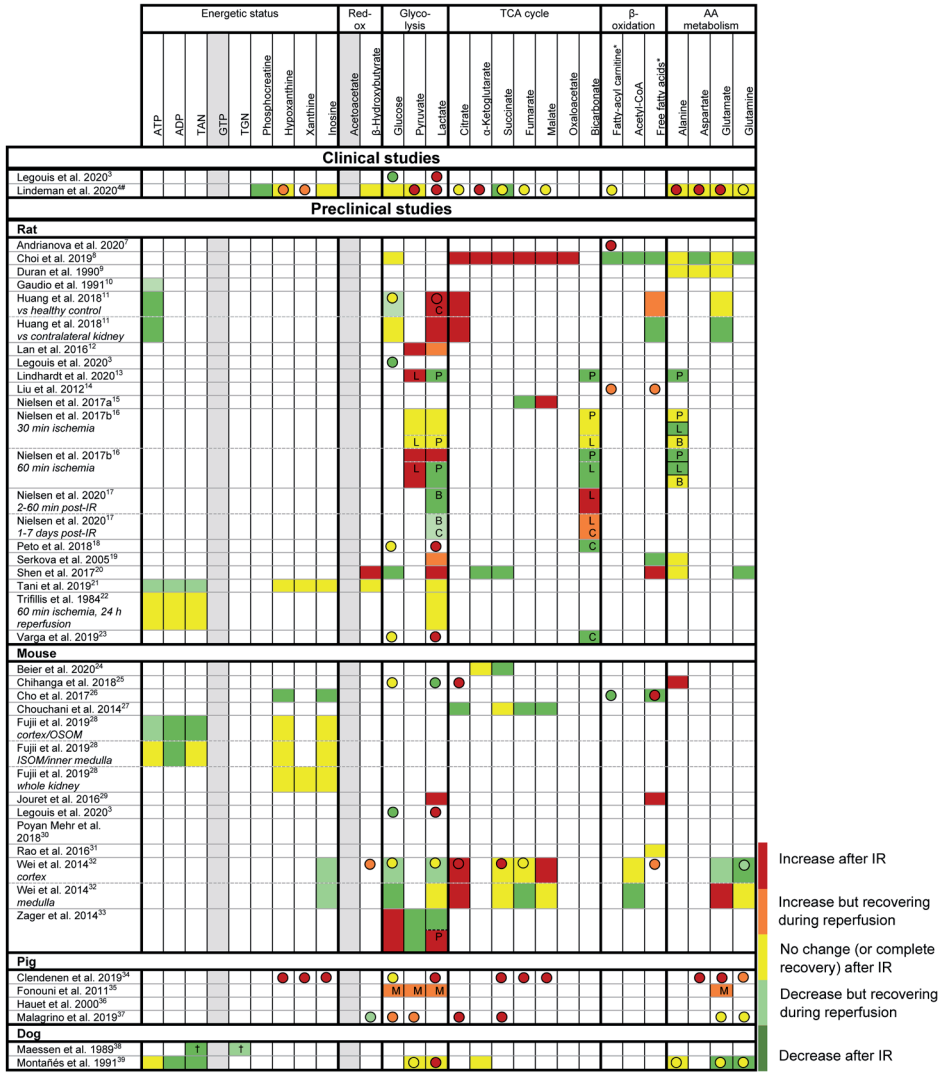


Figure 2. Relative differences in renal tissue (boxes) and blood (spheres, note: often systemic if no data on kidney-specific samples) metabolite levels between control kidneys (healthy/intact control, sham-surgery control, or contralateral kidney: see Table 2) and kidneys after ischemia reperfusion (IR). If no control data was available, data was excluded. Metabolites marked in grey were not reported for tissue or blood samples in any of the preclinical studies. Red = increase after IR. Orange = increase but recovering during reperfusion (requires multiple samples during reperfusion). Yellow = no change (or complete recovery) after IR. Light green = decrease but recovering during reperfusion (requires multiple samples during reperfusion). Green = decrease after IR. TAN: total adenine nucleotides (ATP+ADP+AMP). TGN: total guanine nucleotides (GTP+GDP+GMP). *: variety of fatty acids, results on overall trend. #: additional data on tissue levels is reported in the supplementary data, but no formal statistical evaluation was performed. OSOM: outer stripes of outer medulla. ISOM: inner stripes of outer medulla. †: ischemia time-dependent. P: metabolite/pyruvate ratio. L: metabolite/lactate ratio. B: metabolite/bicarbonate ratio. C: in control (sham-operated animal or contralateral kidney) metabolite levels showed similar dynamics following surgery. M: measured using microdialysis.

Discussion

Recent clinical leads for DGF and AKI point towards a universal discriminatory metabolic profile for, and possibly causal role of, metabolic aspects in renal I/R injury.[3,4] This observation is remarkable considering the obvious mechanistic differences between DGF (warm and cold ischemia) and AKI (exclusively warm ischemia), and may imply a universal mechanism for clinical renal I/R injury. It was reasoned that these observations provide a lead for validation of preclinical models of I/R injury. A systematic review was performed to align the observed metabolic profiles for clinical context with those reported in preclinical studies. This review shows poor alignment of the reported preclinical metabolic data with clinical evidence, and identifies several critical methodological shortcomings in the preclinical studies.

Whilst the phenomenon of I/R injury has been known for over 50 years, a persistent translational gap in transforming the abundant preclinical therapeutic successes towards a clinical benefit remains. In fact, 10 years ago, partners in the NIH CAESAR consortium (in the context of myocardial I/R injury) stated that: "for 40 years, the National Heart, Lung, and Blood Institute has invested enormous resources (at least several hundred million dollars) in preclinical studies aimed at developing infarct-sparing therapies, and several hundred (if not thousands) therapies have been claimed to limit infarct size in preclinical models. Unfortunately, due largely to methodological problems, this enormous investment has not produced any notable clinical application".[1] Similar conclusions were expressed with respect to renal I/R injury.[2,43]

Two recent clinical studies positioned metabolic defects in the center of renal I/R injury.[3,4] Although the conclusions of the studies do not allow discrimination between a causative mechanism or secondary defect, findings are fully discriminatory and provide insight in the early events of clinical I/R injury, and imply a metabolic mechanism as driver of clinical I/R injury. Alignment of the preclinical models with the clinical context identified a number of critical issues that may fundamentally interfere with translation of preclinical findings.

A first, fundamental aspect concerns the diagnosis/definition 'I/R injury'. Clinical studies not only identified a clear signature for I/R injury, but also indicated graded degrees of recovery in the absence of I/R injury, i.e. in the context of transplantation an almost instantaneous functional and metabolic recovery for living donor grafts versus a more suspended recovery for deceased donor grafts (Figure 1).[4] Although less detailed information was available for AKI, different grades of AKI also associate with graded metabolic recoveries (e.g. KDIGO stage 3, demonstrating more severely impaired metabolism compared to less severe, i.e. KDIGO stage 1, injury).[3]

Clinical I/R injury (DGF) was accompanied by a fully discriminatory reperfusion metabolome, an aspect that allowed for discrimination between I/R injury and IR damage. To be specific: I/R injury is the injury triggered by the metabolic paralysis, with persistent ATP catabolism despite adequate reperfusion. IR *damage* on the other hand, reflects tissue damage caused by entry of cellular and/or humoral effectors upon reestablishment of blood flow, processes that are independent from the metabolic cataplexy and may occur in response to an ischemic insult as well as I/R injury.

A further aspect identified in the review were the notable variations in techniques applied to induce IR, ischemia times, timing of the post-reperfusion sampling, and considerable heterogeneity concerning species, strains and gender used. Considering the established interspecies, -strain, and sex variations in metabolism, the metabolic susceptibility, [44-47] and the impact of environmental factors on metabolic flexibility,[7,48,49] this heterogeneity may contribute to the compromised interchangeability of research findings.

Most (23/35) preclinical reports relied on serum creatinine as functional read-out of kidney function/injury. Group-wise comparisons (intervention versus control), and single or short (≤24 hours) follow-up times were generally applied. Consequently, appropriate ischemic controls and/or recovery profiles series required for discrimination between IR damage and injury were absent in preclinical studies. Moreover, conclusions with respect to creatinine clearance from preclinical studies that relied on cardiac arrest, bilateral clamping, or on unilateral clamping/transplantation with unilateral nephrectomy are potentially interfered by a secondary prerenal kidney insufficiency resulting from a uremia-induced somnolence with suppressed thirst reflex. Potential secondary prerenal kidney insufficiency is avoided in protocols applying unilateral clamping without contralateral nephrectomy (14/35 studies) (which obviously interferes with the use of creatinine clearance as read-out) or possibly by renal replacement therapy (none of the identified studies). No study applied prespecified serum creatinine thresholds to grade the degree of injury, or corrected for over- or dehydration during or following surgery.
A broad palette of histological, plasma/serum, and/or urinary markers was used to further grade kidney injury. Since multiple evaluations concluded that histological examinations poorly predict outcome,[50,51] the question arises whether a strong reliance on histology is justified; in fact, one preclinical study reported I/R injury based on serum creatinine levels in absence of histological damage.[28]
Reliance on, or the inclusion of, urinary samples in some studies is remarkable, considering that transient anuria is a key characteristic of clinical renal I/R injury. Consequently, it is unlikely that the injury sustained in these studies reflects the degree of injury in clinical I/R injury.

The majority of preclinical studies (23/27) that include plasma/serum measurements exclusively relied on peripheral samples for metabolic profiling (Table 2). Interpretation of this data is potentially interfered by the physiologic clearance mechanisms.[4] In fact, metabolic signals are fully absent in the peripheral (arterial) blood samples in the clinical setting, and selective post-renal venous sampling was required for distinctive signals. [4]
Tissue metabolites are reported by 25/35 studies. Although metabolic information from tissue samples can be highly informative, this approach is only appropriate for non-diffusible metabolites; a parallel evaluation of metabolic data from tissue biopsies and renal venous samples indicated wash-out of diffusible metabolic intermediates, with stable tissue contents. [4]

Alignment of reported metabolic profiles identified contrasting findings between preclinical and clinical studies. Whilst clinical I/R injury (DGF) is characterized by energetic impairment that persists beyond the 30-minutes post-reperfusion measurement-window (Table 1)[4], reported data from rodent/dog studies implies reinstatement of energy equivalents following reperfusion. Hence, observations in these models align with the observations for grafts without I/R injury.
Selective arteriovenous sampling identified normoxic glycolysis and impaired lactate handling as key characteristics of clinical renal I/R injury (Table 1).[3,4] Only three experimental studies applied selective venous sampling,[3,34,39] but very limited metabolic analysis was performed.
A specific TCA cycle defect at the level of the oxoglutarate-dehydrogenase complex with selective post-reperfusion release of α-ketoglutarate and impaired recovery of tissue succinate levels was identified in clinical I/R injury (DGF) (Table 1). This finding contrasts with the metabolome of rodent I/R injury, which is reportedly associated with an ischemic accumulation of succinate and its subsequent rapid re-oxidation during reperfusion, paralleled by an oxidative burst.[27] The latter rapidly subsides upon normalization of succinate levels (within 5 minutes of reperfusion).[27] This brief insult contrasts with the prolonged, but reversible metabolic defects in clinical I/R injury (Table 1, Figure 1). Although it cannot be excluded that a succinate-driven mechanism contributes to the initiation of I/R injury, the transient character and the apparent rapid metabolic recovery contrast with the clinical context.

In conclusion, this systematic evaluation based on reported metabolic aspects of I/R injury demonstrates profound methodological variability and shortcomings in the preclinical studies. Whilst some of these limitations are inherent to preclinical research, e.g. overall interspecies differences and differences in resilience, most shortcomings can be circumvented. A key issue in preclinical studies is the inability to discriminate between IR damage and I/R injury. Additional challenges in preclinical research are the strong impact of age and comorbidities on incidental AKI and DGF[52-54] (an aspect that is largely

ignored in preclinical studies that generally use young and healthy animals), the need for kidney-specific sampling, prolonged follow-up and bridging renal support to quantify the actual impact of IR. Finally, reported metabolic aspects are diffuse and often scattered. Consequently, studies to date do not allow for adequate metabolic phenotyping and/or comparison of outcomes. To fully capture the complex metabolic interactions, animal models are essential for research. Recommendations to improve the translatability of preclinical research are therefore summarized in Table 5 and Figure 3.

This systemic review has some limitations. The available data is limited. Only two clinical studies are available, one concerning DGF and one on AKI. Although there is consensus that they both reflect renal I/R injury, these are obviously distinct entities. Preclinical reports were extremely heterogenous, hence no formal meta-analysis could be performed. This study is kidney-focused; given the organ-specific difference in metabolism and metabolic rates, observations may not directly translate to other tissues.

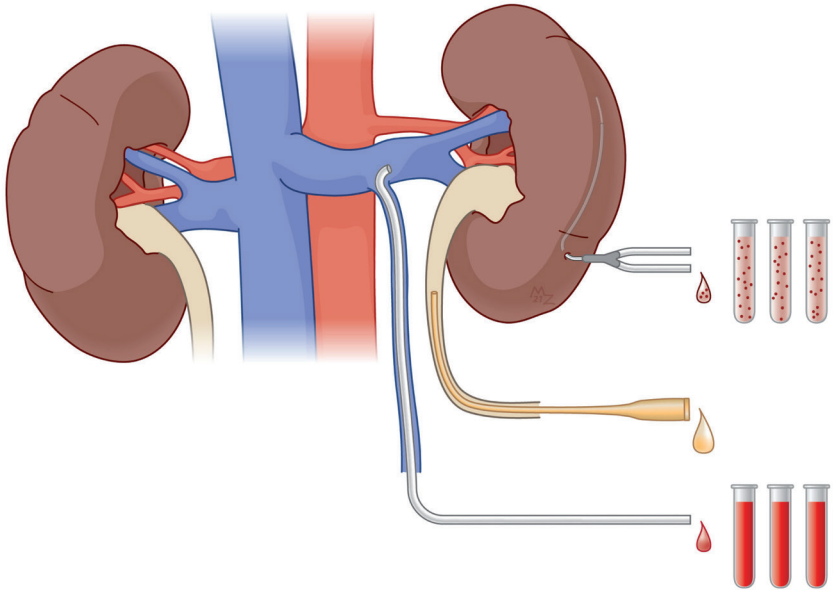


Figure 3. Recommendations to improve the translatability of preclinical models of renal ischemia reperfusion (IR) injury. Ischemic injury should be induced unilaterally in order to avoid interference caused by uremia. Appropriate discrimination between IR damage and IR injury critically relies on the organ-specific assessment of metabolic competence (i.e. prolonged normoxic glycolysis, see outline in Figure 1). Kidney-specific metabolic profiling of the injured kidney can be achieved through renal-vein specific blood sampling using the spermatic vein as access.[39,55] Microdialysis is a potential alternative to arteriovenous sampling, but has not yet been validated for this purpose. Ureterostomy[56] allows for selective functional monitoring of the injured kidney. The model critically relies on optimized ischemia times in order to achieve actual IR injury and avoid excess incidences of IR damage or non-function. It is anticipated that successful implementation of these prerequisites relies on use of rats or larger laboratory animals. Illustration by Manon Zuurmond.

Table 5. Recommendations for future preclinical studies investigating renal metabolism following ischemia reperfusion (IR).

Recommendation	Rationale
Functional IR injury definition	Consensus on renal dysfunction
Renal replacement therapy	Exclusion of prerenal causes of renal IR injury
Optimal ischemia time (titration of IR injury)	Chance for both transient dysfunction, i.e. DGF, and ischemic controls, i.e. primary function
Representative study population	Higher incidence of clinical renal IR injury in older patients
Renal vein catheterization (excludes use of mice)	Arteriovenous kidney-specific sampling; metabolite diffusion
Successive sampling	Monitoring dysfunction and functional recovery
Extended follow-up time	Monitoring dysfunction and functional recovery
Predefined set of metabolites	Insight in metabolic competence, straightforward comparisons

Supplemental data

1.1 Literature search

Two separate systematic literature searches were performed in the databases of PubMed, EMBASE and Web of Science, in which the literature until December 2020 was examined.

Studies were excluded if 1) no preclinical (i.e. animal) model was applied; 2) only organs other than kidneys were examined; 3) only ischemia was applied, in the absence of reperfusion; 4) data was solely gathered after ischemia rather than after IR; 5) only metabolite levels of compounds that are not directly involved in energy metabolism were measured (e.g. phospholipids, drug metabolites); 6) metabolites were not measured (e.g. only enzyme activities); 7) kidney injury was due to other causes than IR (e.g. rejection, toxins); 8) it concerned a review, book section or case report; 9) language was other than English.

Altogether, 35 preclinical studies were included from the systematic searches (Supplementary Figure 1a and 1b) and were subsequently clustered according to their study population (i.e. species studied). Details of the searches in PubMed, EMBASE and Web of Science are given under *1.2 Search components*.

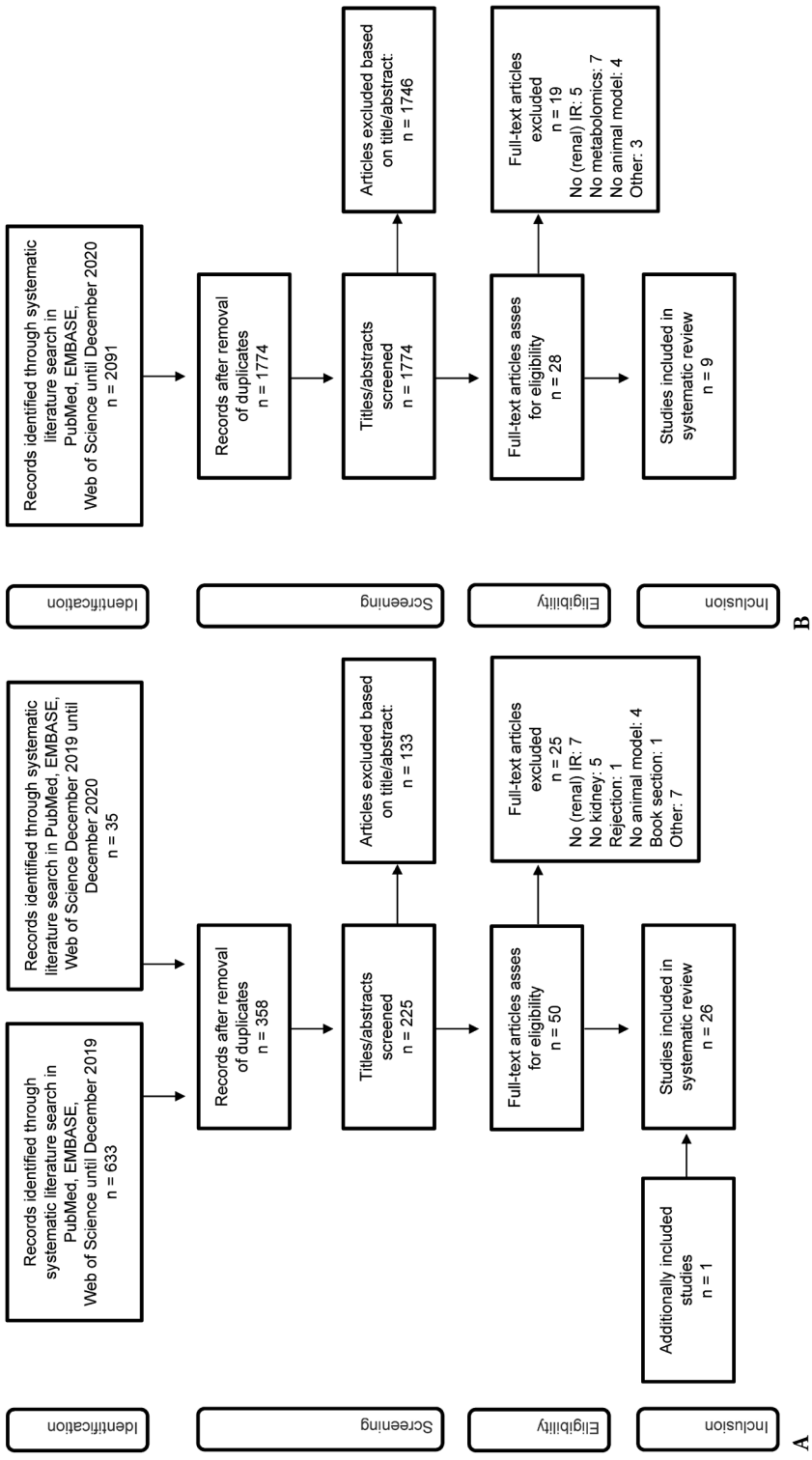
1.2 Search components

1.2.1 DGF search

In total, 220, 15 and 88 unique references were found using systematic searches in respectively Pubmed, EMBASE and Web of Science on the 19th of December, 2019, excluding duplicates. Additionally, 22, 5 and 8 unique references were found using systematic searches in respectively Pubmed, EMBASE and Web of Science on the 1st of December, 2020, excluding duplicates as well as references identified by the DGF search from December 2019.

PubMed

((“ischemia-reperfusion”[tw] OR ischemia-reperfus[tw] OR “ischaemia-reperfusion”[tw] OR ischaemia-reperfus*[tw] OR “Reperfusion Injury”[Mesh:noexp] OR “reperfusion injury”[tw] OR “reperfusion injuries”[tw] OR reperfusion injur*[tw] OR “reperfusion damage”[tw] OR reperfusion damag*[tw] OR “ischemic injury”[tw] OR “ischemic injuries”[tw] OR ischemic injur*[tw] OR “ischaemic injury”[tw] OR “ischaemic injuries”[tw] OR ischaemic injur*[tw] OR “Primary Graft Dysfunction”[mesh] OR “primary graft dysfunction”[tw] OR “primary graft failure”[tw] OR “Delayed Graft Function”[Mesh] OR “delayed graft function”[tw]) AND (“Kidney”[mesh] OR “kidney”[tw] OR “kidneys”[tw] OR “renal”[tw] OR “Kidney transplantation”[mesh] OR kidney transplant*[tw] OR “renal transplantation”[tw] OR renal transplant*[tw] OR kidney graft*[tw] OR renal graft*[tw] OR “renal injury”[tw] OR “renal injuries”[tw] OR “kidney injury”[tw] OR “kidney injuries”[tw]) AND (“Metabolomics”[Mesh]*



Supplemental Figure 1. Flowchart of the systematic literature search performed to identify preclinical studies reporting metabolic data following renal ischemia reperfusion (IR) in the context of a) Delayed Graft Function (DGF) and b) Acute Kidney Injury (AKI).

OR "Metabolome"[mesh] OR "metabonomics"[tw] OR "metabolomics"[tw] OR metabonom[tw] OR metabolom*[tw] OR "metabolic profile"[tw] OR "metabolic profiles"[tw] OR "metabolites"[tw] OR "metabolite"[tw] OR "metabolic"[ti]) AND ("english"[la] OR "dutch"[la] OR "russian"[la]))*

EMBASE

(("ischemia-reperfusion".ti,ab OR "ischemia-reperfus".ti,ab OR "ischaemia-reperfusion".ti,ab OR ischaemia-reperfus*.ti,ab OR *"Reperfusion Injury"/ OR "reperfusion injury".ti,ab OR "reperfusion injuries".ti,ab OR "reperfusion injur*".ti,ab OR "reperfusion damage".ti,ab OR reperfusion damag*.ti,ab OR "ischemic injury".ti,ab OR "ischemic injuries".ti,ab OR ischemic injur*.ti,ab OR "ischaemic injury".ti,ab OR "ischaemic injuries".ti,ab OR ischaemic injur*.ti,ab OR *"Primary Graft Dysfunction"/ OR "primary graft dysfunction".ti,ab OR "primary graft failure".ti,ab OR *"Delayed Graft Function"/ OR "delayed graft function".ti,ab) AND (exp *"Kidney"/ OR "kidney".ti,ab OR "kidneys".ti,ab OR "renal".ti,ab OR exp "Kidney transplantation"/ OR kidney transplant*.ti,ab OR "renal transplantation".ti,ab OR renal transplant*.ti,ab OR kidney graft*.ti,ab OR renal graft*.ti,ab OR "renal injury".ti,ab OR "renal injuries".ti,ab OR "kidney injury".ti,ab OR "kidney injuries".ti,ab) AND (*"Metabolomics"/ OR *"Metabolome"/ OR "metabonomics".ti,ab OR "metabolomics".ti,ab OR metabonom*.ti,ab OR metabolom*.ti,ab OR "metabolic profile".ti,ab OR "metabolic profiles".ti,ab OR "metabolites".ti,ab OR "metabolite".ti,ab OR "metabolic".ti) AND ("english".la OR "dutch".la OR "russian".la))*

Web of Science

(ti=("ischemia-reperfusion" OR "ischemia-reperfus" OR "ischaemia-reperfusion" OR "ischaemia-reperfus*" OR "Reperfusion Injury" OR "reperfusion injury" OR "reperfusion injuries" OR "reperfusion injur*" OR "reperfusion damage" OR "reperfusion damag*" OR "ischemic injury" OR "ischemic injuries" OR "ischemic injur*" OR "ischaemic injury" OR "ischaemic injuries" OR "ischaemic injur*" OR "Primary Graft Dysfunction" OR "primary graft dysfunction" OR "primary graft failure" OR "Delayed Graft Function" OR "delayed graft function") AND ts=("Kidney" OR "kidney" OR "kidneys" OR "renal" OR exp "Kidney transplantation" OR "kidney transplant*" OR "renal transplantation" OR "renal transplant*" OR "kidney graft*" OR "renal graft*" OR "renal injury" OR "renal injuries" OR "kidney injury" OR "kidney injuries") AND (ts=("Metabolomics" OR "Metabolome" OR "metabonomics" OR "metabolomics" OR metabonom* OR metabolom* OR "metabolic profile" OR "metabolic profiles" OR "metabolites" OR "metabolite") OR ti="metabolic") AND la=("english" OR "dutch" OR "russian")) OR (ts=("ischemia-reperfusion" OR "ischemia-reperfus*" OR "ischaemia-reperfusion" OR "ischaemia-reperfus*" OR "Reperfusion Injury" OR "reperfusion injury" OR "reperfusion injuries" OR "reperfusion injur*" OR "reperfusion damage" OR "reperfusion damag*" OR "ischemic injury" OR "ischemic injuries" OR "ischemic injur*" OR "ischaemic injury" OR "ischaemic injuries" OR "ischaemic injur*" OR "Primary Graft*

Dysfunction" OR "primary graft dysfunction" OR "primary graft failure" OR "Delayed Graft Function" OR "delayed graft function") AND ti=("Kidney" OR "kidney" OR "kidneys" OR "renal" OR exp "Kidney transplantation" OR "kidney transplant" OR "renal transplantation" OR "renal transplant*" OR "kidney graft*" OR "renal graft*" OR "renal injury" OR "renal injuries" OR "kidney injury" OR "kidney injuries") AND (ts=("Metabolomics" OR "Metabolome" OR "metabonomics" OR "metabolomics" OR metabonom* OR metabolom* OR "metabolic profile" OR "metabolic profiles" OR "metabolites" OR "metabolite") OR ti="metabolic") AND la=("english" OR "dutch" OR "russian"))*

1.2.2 AKI search

In total, 1662, 92 and 20 unique references were found using systematic searches in respectively Pubmed, EMBASE and Web of Science on the 9th of December, 2020, excluding duplicates as well as references identified in the DGF search.

PubMed

(("Acute Kidney Injury"[majr] OR "Acute Kidney Injury"[ti] OR "Acute Kidney Injuries"[ti] OR "Acute Renal Injury"[ti] OR "Acute Renal Injuries"[ti] OR "Acute Kidney Failure"[ti] OR "Acute Kidney Insufficiency"[ti] OR "Acute Renal Failure"[ti] OR "Acute Renal Insufficiency"[ti] OR "AKI"[ti]) AND ("Metabolomics"[majr] OR "Metabolome"[majr] OR "metabonomics"[ti] OR "metabolomics"[ti] OR metabonom[ti] OR metabolom*[ti] OR "metabolic profile"[ti] OR "metabolic profiles"[ti] OR "metabolites"[ti] OR "metabolite"[ti] OR "Metabolism"[majr:noexp] OR "metabolism"[ti] OR "metabo*"[ti] OR ("Acute Kidney Injury/metabolism"[majr] AND "metabolism"[Subheading:NoExp])) AND ("english"[la] OR "dutch"[la] OR "russian"[la]) NOT (("Cisplatin"[Mesh] OR "cisplatin"[tw] OR "Sepsis"[Mesh] OR "sepsis"[tw] OR "toxic kidney"[tw] OR "toxic renal"[tw] OR "nephrotox*"[tw]) NOT ("surgery"[subheading] OR "Surgical Procedures, Operative"[Mesh] OR "surgery"[tw] OR "surgical"[tw] OR "surgical*"[tw] OR "Perioperative Period"[Mesh] OR "Perioperative Care"[Mesh] OR "peri operative"[tw] OR "perioperative"[tw] OR "Postoperative Period"[Mesh] OR "Postoperative Complications"[Mesh] OR "post operative"[tw] OR "postoperative"[tw] OR "Endovascular Procedures"[Mesh] OR "endovascular"[tw] OR "endo vascular"[tw])) NOT (("Review"[ptyp] OR "review"[ti]) NOT ("Clinical Study"[ptyp] OR "trial"[ti] OR "RCT"[ti] OR "Case Reports"[ptyp] OR "case report"[ti])))*

EMBASE

(("Acute Kidney Failure"/ OR "Acute Kidney Injury".ti OR "Acute Kidney Injuries".ti OR "Acute Renal Injury".ti OR "Acute Renal Injuries".ti OR "Acute Kidney Failure".ti OR "Acute Kidney Insufficiency".ti OR "Acute Renal Failure".ti OR "Acute Renal Insufficiency".ti OR "AKI".ti) AND (exp "Metabolomics"/ OR exp "Metabolome"/ OR "metabonomics".ti OR "metabolomics".ti OR metabonom.ti OR metabolom*.ti OR "metabolic profile".ti OR "metabolic profiles".ti OR "metabolites".ti OR "metabolite"/ OR "metabolite".ti OR*

"Metabolism"/ OR "metabolism".ti OR "metabo".ti) AND ("english".la OR "dutch".la OR "russian".la) NOT ((exp *"Cisplatin"/ OR "cisplatin".ti OR exp "Sepsis"/ OR "sepsis".ti OR "toxic kidney".ti OR "toxic renal".ti OR "nephrotox*".ti) NOT (exp "surgery"/ OR "su".fs OR "surgery".ti,ab OR "surgical".ti,ab OR "surgical*".ti,ab OR exp "Perioperative Period"/ OR "peri operative".ti,ab OR "perioperative".ti,ab OR exp "Postoperative Period"/ OR exp "Postoperative Complication"/ OR "post operative".ti,ab OR "postoperative".ti,ab OR "Endovascular Surgery"/ OR "endovascular".ti,ab OR "endo vascular".ti,ab)) NOT ((exp "Review"/ OR "review".ti) NOT (exp "Clinical Study"/ OR exp "Clinical trial"/ OR "trial".ti OR "RCT".ti OR "Case Report"/ OR "case report".ti)))*

Web of Science
(ti=("Acute Kidney Failure" OR "Acute Kidney Injury" OR "Acute Kidney Injuries" OR "Acute Renal Injury" OR "Acute Renal Injuries" OR "Acute Kidney Failure" OR "Acute Kidney Insufficiency" OR "Acute Renal Failure" OR "Acute Renal Insufficiency" OR "AKI") AND ti=("Metabolomics" OR "Metabolome" OR "metabonomics" OR "metabolomics" OR metabonom OR metabolom* OR "metabolic profile" OR "metabolic profiles" OR "metabolites" OR "metabolite" OR "metabolite" OR "Metabolism" OR "metabolism" OR "metabo*") AND la=("english" OR "dutch" OR "russian") NOT (ti=("Cisplatin" OR "cisplatin" OR "Sepsis" OR "sepsis" OR "toxic kidney" OR "toxic renal" OR "nephrotox*") NOT ts=("surgery" OR "surgery" OR "surgical" OR "surgical*" OR "Perioperative Period" OR "peri operative" OR "perioperative" OR "Postoperative Period" OR "Postoperative Complication" OR "post operative" OR "postoperative" OR "Endovascular Surgery" OR "endovascular" OR "endo vascular")) NOT ((TI="Review" OR DT="review") NOT TI=("Clinical Study" OR "Clinical trial" OR "trial" OR "RCT" OR "Case Report" OR "case series")))*

1.3 Data collection

One author collected data from the included studies. Extracted data included specifics concerning the study populations, control groups, intervention techniques, sampling techniques, measurement techniques, applied definitions of I/R injury, as well as metabolic data (i.e. reported metabolite levels). If controls were missing, metabolic data was excluded. To present the metabolic data of the studies, reported means of measured metabolite levels in study groups and control groups within single studies were collected and compared. All extracted data is publicly available in the cited publications.

1.4 Synthesis of results

Studies were considered eligible for inclusion of outcomes if they reported metabolic aspects, which is also a prerequisite according to the inclusion criteria. The data (i.e. reported metabolite levels) were mapped along theoretical pathways (energetic status, redox, glycolysis, TCA cycle, β-oxidation, amino acid metabolism) to obtain insight into changes in the major metabolic routes. Absolute measures were converted to semi-

quantitative data (i.e. dynamics of changes), which enabled direct comparisons between studies, despite great variety in applied methods. The studies were clustered according to their study population (i.e. type of animal studied). Altogether, this resulted in an illustrative overview in the form of a heatmap.

1.5 Risk of bias and sensitivity analysis of results

In this systematic review, we did not assess risk of bias of the included preclinical studies, as this is inherent to preclinical studies and their great heterogeneity (i.e. differences in species, strains, induction of injury). This excessive heterogeneity and concomitant granulated data also restricted the assessment of robustness of results, and results were therefore converted to qualitative aspects in a theoretical framework.

1.6 Statistics

This systematic review is a qualitative and descriptive study, and the reported quantitative data has already been statistically examined in the original publications. Therefore, further statistical tests were not applied.

1.7 Other information

The review was not registered. A review protocol was not prepared, as this study concerns an explorative meta-analysis. The PRISMA checklist for systematic reviews is given in Supplementary Table 1.

Acknowledgements

We would like to acknowledge LUMC librarian Jan W. Schoones for his dedicated support concerning the development of the literature search queries.

Supporting Table 1. PRISMA guidelines for systematic reviews checklist.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Supp. Info 1.1
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	5
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supp. Info 1.2
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	5
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Supp. Info 1.3
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Supp. Info 1.3
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Supp. Info 1.3
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	NA
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Supp. Info 1.3

Section and Topic	Item #	Checklist item	Location where item is reported
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Supp. Info 1.4
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Supp. Info 1.4
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Supp. Info 1.4
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Supp. Info 1.4
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Supp. Info 1.4
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	NA
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	10-16
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	10-16
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	6, Supp. Info 1.1-1.2
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Supp. Info Fig. 1
Study characteristics	17	Cite each included study and present its characteristics.	Table 2+3
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Supp. Info 1.5
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Fig. 1

Section and Topic	Item #	Checklist item	Location where item is reported
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	10-16
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Supp. Info 1.6
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	6-9
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Supp. Info 1.5
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	10-16
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	NA
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	7-11
	23b	Discuss any limitations of the evidence included in the review.	16
	23c	Discuss any limitations of the review processes used.	16
	23d	Discuss implications of the results for practice, policy, and future research.	10-16
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Supp. Info 1.7
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	5
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	NA
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	16
Competing interests	26	Declare any competing interests of review authors.	16
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Supp. Info 1.3

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Chapter 7

Circumventing the Crabtree effect in cell culture: a systematic review

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Abstract

Metabolic reprogramming and mitochondrial dysfunction are central elements in a broad variety of physiological and pathological processes. While cell culture established itself as a versatile technique for the elaboration of physiology and disease, studying metabolism using standard cell culture protocols is profoundly interfered by the Crabtree effect. This phenomenon refers to the adaptation of cultured cells to a glycolytic phenotype, away from oxidative phosphorylation in glucose-containing medium, and questions the applicability of cell culture in certain fields of research. In this systematic review we aim to provide a comprehensive overview and critical appraisal of strategies reported to circumvent the Crabtree effect.

Introduction

Metabolic flexibility is the ability to respond or adapt to changes in energy supply and demand under a variety of conditions, and is a prerequisite for optimal cellular function, proliferation, differentiation, and survival.[1,2] Whilst this adaptive capacity (e.g. increasing the reliance on glycolysis) is a critical aspect in many physiological processes, ranging from exercise metabolism to cell fate determination and immunometabolism, metabolic inflexibility is increasingly linked to a wide range of chronic diseases such as type 2 diabetes, obesity and Alzheimer's disease. [1, 3-6]

The most well-known example of metabolic reprogramming is the Warburg phenomenon, a condition characterized by a deviation of the metabolic profile in which cancer cells essentially rely on glycolysis instead of oxidative phosphorylation (OXHPOS).[7] A parallel metabolic phenomenon, referred to as the Crabtree effect,[8] has been described in cultured cells. The Crabtree effect describes the phenomenon that cells cultured in glucose-containing culture medium adapt to a glycolytic phenotype, despite the presence of oxygen and functional mitochondria.[8,9] This phenomenon has major consequences for cell culture-based studies that aim at addressing aspects of the physiologic metabolic flexibility and/or studying mitochondrial processes. In fact, failure to address this point may lead to misinterpretation of conclusions such as 'dysfunctional' energy metabolism (metabolic inflexibility) or illusory cellular resistance to mitochondrial toxicants and drugs which diminishes the prediction of potential drug toxicity in vivo.[10]

Consequently, in order to adequately study the aspects of metabolic flexibility and/or mitochondrial processes in cell culture, it is crucial to restore the cellular physiologic and metabolic state by reverting the cellular metabolism from glycolysis-dominated back to OXPHOS-dominated. Therefore, the aim of this systematic review was to provide a systematic, comprehensive overview and critical appraisal of reported strategies that circumvent the Crabtree effect. Molecular aspects underlying the Crabtree effect were considered beyond the scope of this review.

Materials and methods

In order to identify relevant studies reporting on strategies circumventing the Crabtree effect in cell culture, two complementary search strategies were designed: a systematic literature search and a citation search. The systematic literature search was conducted in PubMed, EMBASE and Web of Science. The following keywords, or synonyms thereof, were included: Crabtree effect, cell culture, glycolysis, OXPHOS, metabolic switch. This systematic literature search was supplemented with a citation search in Web of Science.

Herein, all articles were identified that referred to the original article of H.G. Crabtree,[8] and specifically focused on cultured cells. Both search strategies were conducted on June, 2nd, 2020 and were performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[11] Details of both search strategies are provided in the Supplemental Data: https://figshare.com/s/195511957a78bfbfee4f.

All titles and abstracts were screened by two authors (MDK and AS) to identify relevant studies (Figure 1). Discrepancies in article eligibility were resolved by joint review and consensus. Subsequently, full text articles were assessed for eligibility. Studies that were not published in English or Dutch, and studies that were not conducted in an in-vitro setting were excluded. Also articles focusing on yeast, embryonic or cancer cells were excluded.

From the included articles, the following data were extracted: year of publication, cell type, initial cell culture medium, intervention strategy to circumvent the Crabtree effect, whether the intervention is induced or performed under continuous control, duration of intervention, and author's key findings.

Results

Strategies circumventing the Crabtree effect

The systematic literature and citation search identified 950 unique references. Of these articles, 25 articles aimed at restoring OXPHOS dominance for adenosine triphosphate (ATP) synthesis under cell culture conditions (Figure 1). The described intervention strategies reverting the Crabtree effect can be broadly classified into four categories: (1) adjustment of the cell culture medium, (2) the use of glycolytic inhibitors, (3) strategies that target mitochondria, and (4) miscellaneous interventions. The respectively characteristics and key findings of each article are summarized in Tables 1-4. A graphical summary of reported strategies aimed at reverting the Crabtree phenomenon is provided in Figure 2.

Culture medium adjustments

The most extensively reported strategy to circumvent the Crabtree effect is replacement of the standard, high glucose (10-25mM) cell culture medium, by a medium with (sub) physiologic glucose concentrations or no glucose at all (Table 1).

Lowering glucose concentrations

Several authors explored whether a reduction in glucose concentrations promoted a metabolic shift from glycolysis to OXPHOS.[9, 12-18] Remarkably, it was shown that the Crabtree effect is not restricted to cells kept in high glucose medium (10-25 mM), but

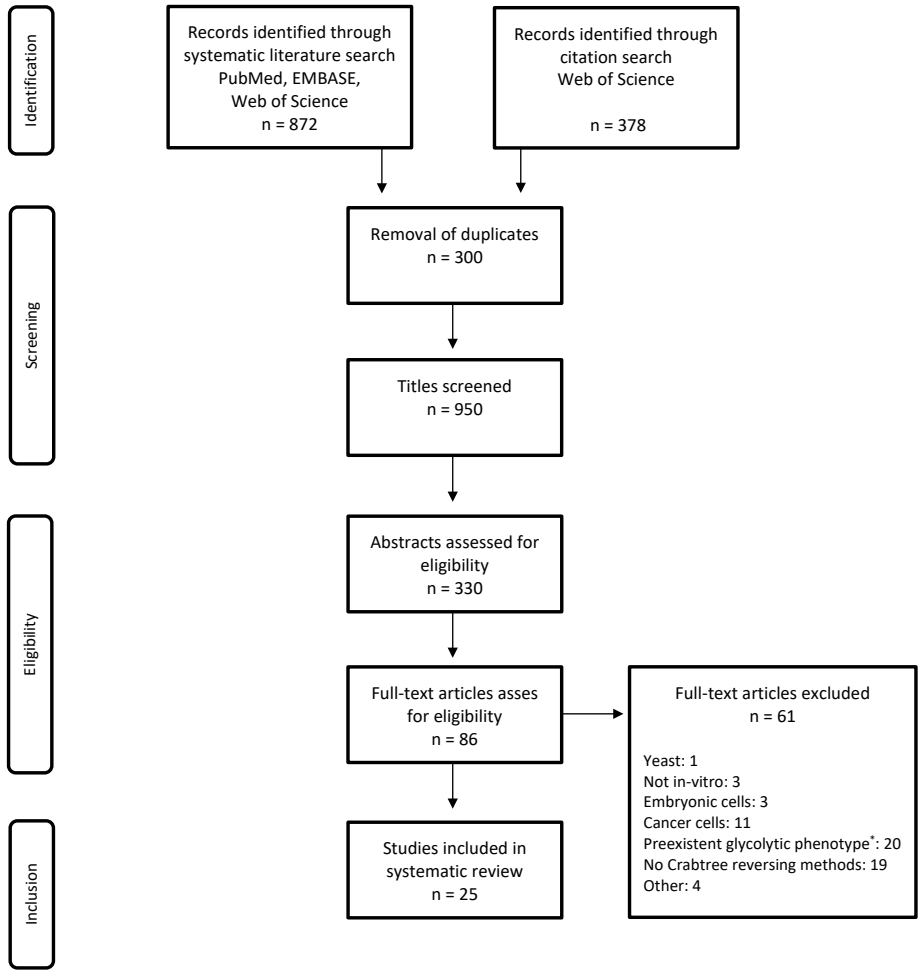

Figure 1. PRISMA flow diagram of the systematic literature search.
*The 20 articles that fall into this category concern articles in which the glycolytic phenotype is already part of the physiological response. For instance, the switch from oxidative phosphorylation to glycolysis is a hallmark of several cell types, including mesenchymal stem cells undergoing osteogenic differentiation, IL-33 activated mast cells, proliferation T cells, nucleus pulposus cells, and dendritic cells during acute activation.

also persist at more physiologic glucose concentrations (5.0-5.6 mM).[12-15] Only when glucose levels were further lowered to concentrations of 0.1-5.0 mM, an increased shift towards OXPHOS was observed in cells.[12,15,16] In this context, Mot et al. and Arend et al. described that reversal of the Crabtree effect only occurred after total glucose depletion: fibroblasts and astrocytes cultured in 2.0-5.0 mM glucose first completely consumed the available glucose to lactate, after which cells were forced to utilize the accumulated lactate as energy substrate via mitochondrial OXPHOS in order to fulfill their energy requirements.[9,18]

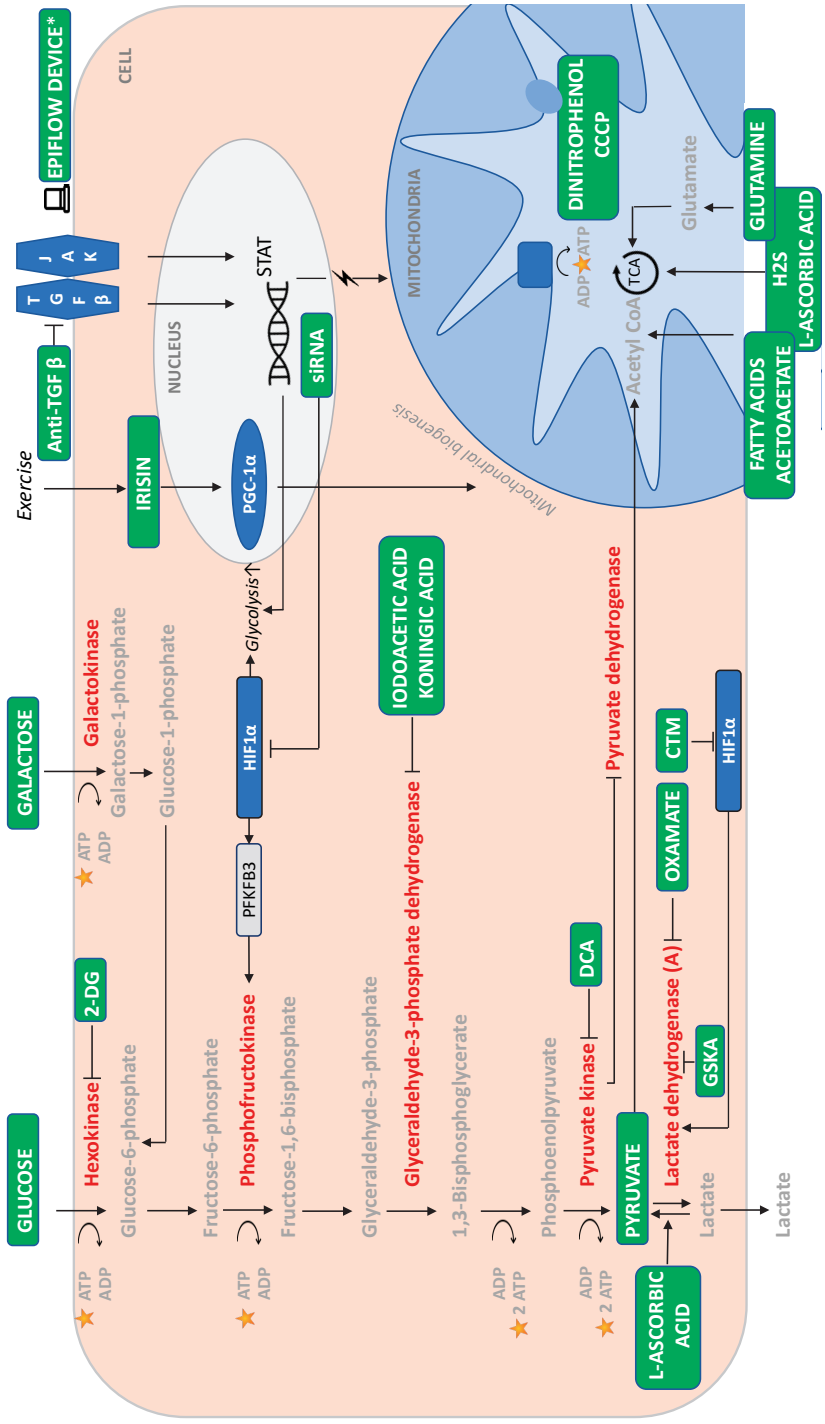

Figure 2. Graphical summary of reported strategies aimed at circumventing the Crabtree effect.

* A cell culture device in which continuous nutrient supply is combined with continuous oxygenation.[36]

2-DG, 2-deoxyglucose; ADP, adenosine diphosphate; AMPK, adenosine monophosphate-activated protein kinase; anti-TGF β, anti-transforming growth factor beta; ATP, adenosine triphosphate; CCCP, carbonyl cyanide-p-chlorophenylhydrazone; DCA, dichloroacetate; HIF1α, hypoxia-inducible factor 1α; mTOR, mammalian target of rapamycin; TCA, tricarboxylic acid cycle.

Table 1. Summary of studies aimed at circumventing the Crabtree effect by adjustment of the cell culture medium. *ATP, adenosine triphosphate; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; ECAR, extracellular acidification rate; FBS, fetal bovine serum; FCS, fetal calf serum; HIF1α, hypoxia-inducible factor 1α; LDH, lactate dehydrogenase; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; RPTC, renal proximal tubular cells; TCA, tricarboxylic acid cycle.*

	Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author–Year
Cell culture medium interventions	Non-transformed human fibroblasts	Glucose medium (conc. glucose not specified), 2mM glutamine, 10% dialyzed FCS.	Medium **without hexose,** with glutamine and 10% dialyzed FCS Galactose medium (**5.5mM galactose**), with glutamine and 10% dialyzed FCS	Incubated Incubated	1 day 1 day	○ "When **deprived of sufficient glucose** in growth medium, cells have been found to increase the supply of energy generated by respiratory metabolism, which can be measured as increased rates of glutamine oxidation." ○ "We have observed **that galactose grown** human cells activate respiratory metabolism to the same degree as cells incubated in **hexose-free medium**."	Kuchka et al. 1981 (19)
	Coronary endothelial cells	Medium 199 (5.6mM glucose), Earle's salts, 20% FCS.	Saline buffer solution, **<1mM glucose**	Incubated	1 hour	○ "Coronary endothelial cells utilize **glucose,** at physiological concentrations, predominantly for glycolytic energy production. The metabolic pattern is characteristic for the **Crabtree** effect." ○ "Glucose oxidation in the Krebs cycle was increased at **glucose** concentrations **lower than 1mM**." ○ "Below 1mM glucose, formation of lactate from glucose decreased."	Krützfeldt et al. 1990 (12)
	Rabbit renal proximal tubular cells	DMEM/F-12 (no glucose or pyruvate), bovine insulin, human transferrin, selenium, hydrocortisone, heptanoate and: **5mM glucose** or 17.5mM glucose or **5mM galactose**. Culture dishes were constantly shaken (SHAKE) or held stationary (STILL).	-	Continued	7 days	○ "All **SHAKE-treated cells** had equivalent lactate levels on day 1, and these levels were less than 50% of their STILL counterparts." ○ "**Lowering** the concentration of **glucose** to a physiologic level (**5mM**) had **no effect** on lactate levels or LDH activity in **SHAKE or STILL** cells." ○ "**Substituting 5mM galactose for 5mM glucose** in the culture medium significantly reduced the lactate content of both SHAKE and STILL RPTEC but had no impact on LDH activity." ○ "**Substitution of glucose with galactose** produced an even greater increase in sensitivity to antimycin A in **SHAKE** cells."	Griner et al. 1994 (13)
	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, **0 or 5mM glucose**, 0.44mM L-alanine, **5mM lactate**, 15mM $NaHCO_3$	-	Continued	12 days	○ "RPTC grown in the presence of **5mM glucose** exhibited net lactate consumption during the first 8 days of culture. However, after 10 and 12 days of culture, lactate metabolism was shifted from net consumption to net production, which suggested the reversion of RPTC metabolism to glycolysis." ○ "In RPTC cultured in the **absence of glucose**, rates of net lactate consumption were equivalent during 12 days of culture."	Nowak et al. 1996 (14) *(Also in Table 4)*

Cell culture medium interventions

	Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author–Year
	Human skin fibroblasts	DMEM (25mM glucose), 4mM glutamine, 1mM pyruvate, 10% FBS.	**10mM galactose**, 1mM pyruvate, 4mM glutamine .	Incubated	3 days	○ "Cells grown in **galactose** exhibit a five- to sixfold decrease in ECAR, reflecting decreased glycolysis, and a twofold increase in the OCR, consistent with a switch to glutamine oxidation." ○ "Cells grown in **galactose-containing medium** maximize mitochondrial ATP production by using a larger fraction of mitochondrial respiration for ATP synthesis."	Gohil et al. 2010 (20)
	Deep and superficial chondrocyte cells	Isolated chondrocytes, DMEM, 1.6mM L-glutamine, 16% FCS.	DMEM, **deprived of** or supplemented with **glucose** (0.5-22mM)	Incubated	Not specified	○ "The OCR in **19mM glucose** was not significantly different to baseline values of **5mM glucose**. The upregulation of oxygen consumption compared to values in **5mM** was significant at glucose concentrations of **below 3mM**." ○ "The oxygen consumption by the superficial and deep cell subpopulations increased progressively with glucose deprivation, rising 2.5-fold as the media glucose was **reduced from 5 to 0.5mM**." "Over 90% of the increase in oxygen consumption with glucose depletion appears to be accounted for by the **oligomycin-sensitive compartment,** that is **oxidative phosphorylation**." ○ "Lactate release was progressively reduced by **increasing glucose deprivation**, indicating that the glycolytic rate of the cells is restricted by limited glucose availability."	Heywood et al. 2010 (15) *(Also in Table 2)*
	L6 cells	DMEM without glucose, **10mM galactose,** 6mM glutamine, 1mM sodium pyruvate, 10% FBS.	-	Continued	Min. 7 days	○ "Investigation into cellular bioenergetics showed that **galactose cultured L6 cells** have a significantly increased OXPHOS capacity compared to glucose cultured cells." ○ "Importantly, cells in glucose were able to up-regulate glycolysis, while **galactose** cells were not." ○ "**Galactose cultured L6 cells** were significantly more sensitive to classical mitochondrial toxicants than glucose cultured cells." ○ "Seahorse extracellular flux analyser demonstrated that OCR was significantly increased whereas ECAR, a measure of glycolysis, was decreased in cells grown in **galactose**."	Dott et al. 2014 (21)
	Bovine chondrocytes	Isolated chondrocytes, DMEM (deprived of glucose), 2mM L-glutamine, 10% FBS and: 10mM glucose (high) or; **1mM glucose (low)** + **9mM galactose.**	-	Continued	4 population doublings	○ "Chondrocytes exhibited significantly greater oxidative phosphorylation in **1mM glucose** compared with **10mM glucose**, both at day 0 and after 4 population doublings." ○ "Cells expanded in **low glucose** derived 57% of their ATP from aerobic metabolism, compared with 23% in high glucose." ○ "If, after 4 population doublings in **low glucose**, chondrocytes were switched to high glucose conditions and vice versa, the metabolic differences observed between the expansion conditions were mostly reversible."	Heywood et al. 2014 (16)

	Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author–Year
Cell culture medium interventions	LLC-PK1 cells	DMEM or DMEM/F-12 (conc. glucose not specified), 10% FBS.	**10mM galactose** medium	Incubated	1 day	dfs ○ "Culturing cells with galactose as an energy source forces kidney tubular epithelial cells to rely on mitochondrial oxidative respiration rather than glycolysis."	Kishi et al. 2015 (22)
	Cardiac progenitor cells	DMEM/F12 (17.5mM glucose), 2.5mM glutamine, 0.5mM pyruvate, 10% embryonic stem cell FBS and supplements.	DMEM without substrates and **0–5–25mM glucose**. DMEM without substrates for 1 hour, followed by addition of **5 mM glucose, 1 mM pyruvate, 4 mM glutamine or 100 μM BSA-palmitate**	Incubated Incubated	4 days ± 40 minutes	exposure of cells to 25 mM Glc resulted in a 40% increase in glycolytic flux compared with cells in 5 mM Glc ($p < 0.01$), and this was inhibited by the GAPDH inhibitor, KA (Fig. 2B) ○ "Exposure of cells to 25mM glucose resulted in a 40% increase in glycolytic flux compared with cells in 5mM glucose." ○ "Glucose, provided as the sole substrate, decreased OCR by up to 50% with maximal responses near the normal plasma levels of glucose. Such glucose-induced decreases in respiration are suggestive of the Crabtree effect." ○ "**Glutamine** increase ATP demand and supported uncoupled respiration." ○ "Although **BSA-palmitate**, by itself, did not increase mitochondrial OCR, the presence of **glutamine** increased OCR by 66% when fatty acids were present, and this stimulation of respiration significantly exceeded that when **glutamine** was provided as the sole substrate." ○ The increase in glycolysis due to glucose treatment was almost completely inhibited by **koningic acid**.	Salabei et al. 2015 (17) *(Also in Table 2)*
	1) Primary adult human fibroblasts 2) Primary mouse brain astrocytes	1) DMEM (25mM glucose), 1mM L-glutamine, 10% FBS.* 2) DMEM (25mM glucose), 10% FBS.**	1. DMEM (3.5mM glucose), 1mM L-glutamine, 9.7mM mannitol, 10% FBS 2. DMEM (5.0mM glucose), 10% FBS	Incubated Incubated	1) 13 days 2) 5 days	○ "**Autonomous depletion** of medium glucose induces a lactate-consuming phase." ○ "**Complete autonomous depletion** of medium glucose forces cells to utilise lactate via mitochondrial OXPHOS to supply their energy needs." ○ "**Autonomous depletion** of medium glucose induces increased sensitivity to the OXPHOS inhibitor rotenone."	Mot et al. 2016 (9) Methods: Garfield 2010 (62) Hare et al. 2013 (63)
	C2C12 mouse myoblasts	DMEM (25mM glucose), 10% FBS.	DMEM-A (**absence of serum, glucose, pyruvate and glutamine**)	Incubated	1 hour	○ "Our findings suggest that the Crabtree effect is decreased in cells subjected to **short-term starvation**, which is possibly associated with the inhibition of the mitochondrial oxidation of glutamine**."**	Zeidler et al. 2017 (23)

	Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author–Year
Cell culture medium interventions	Primary and BV-2 microglial cells	BV-2: DMEM (25mM glucose), 4mM glutamine, 10% FCS. Primary cells: MEM (5.6mM glucose), 4mM glutamine, 10% FCS.	ACSF assay medium, **5mM pyruvate or 2.5mM glutamine**	Incubated	2 hours	○ **Pyruvate**: "when glucose was replaced by **pyruvate**, ATP could not be produced in glycolysis; therefore, to produce ATP, pyruvate should enter into the TCA cycle." "Pyruvate-supported oxidation significantly enhanced the cellular ATP levels in primary cells." ○ **Glutamine**: "under starving conditions, the basal respiration is increased by the addition of **glutamine** as a single metabolic fuel, and the glutamine-induced oxygen-consumption is associated with ATP synthesis in primary and BV-2 microglial cells."	Nagy et al. 2018 (24) *(Also in Table 2)*
	Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs)	Glucose medium: RPMI medium (conc. glucose not specified), GlutaMAX, Gem21. Glucose fatty acids medium: RPMI medium (conc. glucose not specified), GlutaMAX, Gem 21, **50μM Palmitic Acid** and **100 μM Oleic Acid**	Fatty acids only medium: RPMI medium **without glucose**, GlutaMAX, **50μM Palmitic Acid** and **100 μM Oleic Acid**	Incubated	7 days	○ "**Glucose deprivation** results in the inhibition of HIF1α and LDH-A activity, and repression of aerobic glycolysis." ○ "Glucose rich medium promotes glycolytic metabolism, even in the presence of fatty acids whereas media containing **fatty acids** as the only energy source allowed for normal physiological metabolic substrate utilization."	Hu et al. 2018 (25) *(Also in Table 4)*
	Rat astrocytes	DMEM (25mM glucose), 1mM pyruvate, 44.6mM sodium bicarbonate, 10% FCS.	Glucose-free **DMEM**, supplemented with **2mM glucose**, 44.6mM sodium bicarbonate	Incubated	0–15 days	○ "The cells rapidly consumed the available glucose and the culture medium was already within 2 days completely deprived of glucose. This cell-dependent metabolic glucose depletion was accompanied by a rapid increase in the extracellular concentration of lactate." ○ "The **lactate consumption phase** was characterized by a steady decrease in extracellular lactate concentration." ○ "In conclusion, glucose depletion experiments revealed that astrocytes in culture efficiently metabolize **glucose to lactate** via glycolysis and subsequently **utilize the lactate** released for mitochondrial energy production."	Arend et al. 2019 (18)
	LLC-PK1 cells	DMEM/F12 without glucose, supplemented with 5mM glucose, 3% FBS.	DMEM/F12 without glucose, supplemented **with 5mM glucose**, 3% FBS and **5mM acetoacetate.**	Incubated	48 hours	○ "Basal respiration, maximal respiration, spare respiratory capacity and ATP-linked respiration were significantly increased in cells grown in growth medium supplemented with **5mM acetoacetate**. In contrast, glycolytic capacity, as well as glycolytic reserve were significantly reduced in the acetoacetate group."	Denoon et al. 2020 (27)

Replacing glucose by other primary substrates

A second series of studies evaluated the use of glucose-free cell culture medium (Table 1). [13,14,17,19-25] In these studies, the cell culture medium was often enriched with other, exogenous energy substrates such as carbohydrates (galactose), amino-acids (glutamine), fatty acids (palmitate), pyruvate or ketone bodies (acetoacetate).

Most of the glucose-replacement studies evaluated the effect of substituting glucose by galactose in the culture medium.[13,19-22] The rationale behind this strategy is that slow oxidation of galactose to pyruvate yields not sufficient ATP per unit time, and thus impels cells to rely on OXPHOS–mostly by an increased glutaminolysis–to generate ATP for cellular homeostasis.[21,9,26] It was concluded that cells grown in galactose-medium (5.0-10.0 mM), had a lower glycolytic rate, and an increased reliance on OXPHOS.[13,19-22] Furthermore, cells cultured in galactose-enriched medium are more sensitive to the mitochondrial inhibitors rotenone and antimycin A, confirming the increased reliance on mitochondrial respiration to meet energy requirements.[13,21]

A more direct enforcement of OXPHOS dominated ATP synthesis includes the exclusive provision of substrates in glucose-free medium that are metabolized in the tricarboxylic acid cycle (glutamine, pyruvate, acetoacetate and/or fatty acids).

Kuchka et al. showed that human fibroblasts increased glutamine oxidation in hexose-free medium, and hence enhanced OXPHOS.[19] A similar phenomenon was observed in microglial cells.[24]

Similarly, replacement of glucose by pyruvate as the sole substrate increased mitochondrial respiration in microglial cells (note that the conversion from pyruvate to lactate does not generate ATP, and pyruvate is therefore forced to enter the mitochondria via acetyl coenzyme A for ATP production).[24]

A similar phenomenon has been described for human pluripotent stem cell-derived cardiomyocytes, in which simulation of their in vivo preference for β-oxidation by culturing cells in fatty-acids-only medium (deprived of glucose), glycolytic rates reduced and OXPHOS rates increased.[25]

Likewise, supplementation of growth medium (5 mM glucose) with the ketone body acetoacetate–the preferred energy substrate of proximal tubules cells–increased mitochondrial respiration and decreased glycolytic capacity in LLC-PK1 cells.[27]

Glycolytic inhibitors

Conceptually, an obvious approach to prevent excessive aerobic glycolysis in cultured cells, is the inhibition of glycolytic enzymes (Figure 2, Table 2). For example, inhibiting the glycolytic enzyme hexokinase by 2-deoxyglucose (20.0-50.0 mM), effectively reduces glycolytic rates, and increases OXPHOS.[15,28] Alternatively, dichloroacetate (0.1 mM) inhibits pyruvate dehydrogenase kinase and reversed the glycolytic phenotype.[29] Other

Table 2. Summary of studies aimed at circumventing the Crabtree effect with glycolytic inhibitors. *ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle medium; FBS, fetal bovine serum; FCS, fetal calf serum; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.*

	Cells	Initial medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author–Year
Glycolytic inhibitors	Rat proximal tubular epithelial cells	DMEM/F-12 (17.5mM glucose), $NaHCO_3$, 5% FBS.	HBSS, 5% FBS, 0, 5 or 20mM glucose and: **0.03-1mM iodoacetic acid** or DMEM/F12, 5% FBS, **20mM 2-deoxyglucose**	Incubated	1 day	○ "Oxidative respiration could be restored by uncoupling mitochondria with **CCCP** or by inhibition of glycolysis with **iodoacetic acid** and **2-deoxyglucose** which indicates that respiration is inhibited by glycolysis (also known as the Crabtree effect)."	Chi et al. 1995 (28) *(Also in Table 3)*
	Deep and superficial chondrocyte cells	Isolated chondrocytes	5mM glucose, **50mM 2-deoxyglucose**	Incubated	Not specified	○ "Both populations expressed the Crabtree phenomena, with oxygen consumption increasing ±2.5-fold in response to glycolytic inhibition by **glucose deprivation** or **2-deoxyglucose**."	Heywood et al. 2010 (15) *(Also in Table 1)*
	Cerebellar granule cells	Basal Medium Eagle (5.6mM glucose), 5mM KCl, 2mM glutamine, 10% FCS.	**0.1mM dichloroacetate**	Incubated	1 day	○ "Cells subjected to an apoptotic stimulus, activate a Warburg-effect like mechanisms, i.e. suppression of OXPHOS combined with activation of aerobic glycolysis as the main pathway for ATP synthesis." ○ "D**ichloroacetate** was shown to reverse the glycolytic phenotype – which characterizes the early phase in our model of apoptosis– lowering lactate level and raising mitochondrial ROS production, which is associated with the enhanced consumption of oxygen in the OXPHOS reaction."	Bobba et al. 2015 (29)
	Cardiac progenitor cells	DMEM/F12 (17.5mM glucose), 2.5mM glutamine, 0.5mM pyruvate, 10% embryonic stem cell FBS and supplements.	DMEM (without substrates) and 0–5–25mM glucose. DMEM, 1mM L-glutamine, 0–5–25mM glucose, **5μM koningic acid**.	Incubated Incubated	4 days Not specified	exposure of cells to 25 mM Glc resulted in a 40% increase in glycolytic flux compared with cells in 5 mM Glc ($p < 0.01$), and this was inhibited by the GAPDH inhibitor, KA (Fig. 2B) ○ "Exposure of cells to 25mM glucose resulted in a 40% increase in glycolytic flux compared with cells in 5mM glucose." ○ "The increase in glycolysis due to glucose treatment was almost completely inhibited by **koningic acid**."	Salabei et al. 2015 (17) *(Also in Table 1)*
	Primary and BV-2 microglial cells	BV-2: DMEM (25mM glucose), 4mM glutamine, 10% FCS. Primary cells: MEM (5.6mM glucose), 4mM glutamine, 10% FCS.	ACSF assay medium, 10mM glucose, **10mM sodium oxamate**	Incubated	± 1.5 hours	○ "Inhibition of lactate dehydrogenase by **oxamate** stimulated the entry of glycolytic pyruvate into mitochondria, increased cellular respiration and decreased the rate of acidification in both primary and BV-2 microglial cells."	Nagy et al. 2018 (24) *(Also in Table 1)*

glycolytic inhibitors reported to reverse the Crabtree effect include iodoacetic acid (0.03-1.0 mM) and koningic acid (5.0 μM) through inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase.[17,28] Also inhibition of lactate dehydrogenase (LDH) by 10.0 mM sodium oxamate reduced lactate accumulation, and – as described by the authors – 'slightly' (significance was not provided) enhanced cellular respiration due to an increased entry of pyruvate into mitochondria.[24]

Mitochondrial interventions

Four identified studies assessed the potential of mitochondrial uncouplers (Table 3) and demonstrated that the proton motive force needed to generate ATP by ATP synthase is dissipated, causing mitochondrial respiration to be maximal.[28,30-32] Three studies conducted in the seventies, showed that the glucose-induced depression of oxygen respiration in rat lung cells and heart muscle cells, was prevented by the uncoupler dinitrophenol (0.025-0.25 mM).[30-32] In 1995, Chi et al. concluded that uncoupling the mitochondria with 4.0 μM carbonyl cyanide-p-chlorophenylhydrazone restored oxidative respiration in rat proximal tubular epithelial cells.[28] A critical question, however, is whether the increased uncoupled respiration indicates circumvention of the Crabtree effect because the uncoupled maximal oxygen consumption rates do not contribute to mitochondrial ATP generation.

Miscellaneous strategies

Several indirect strategies (Table 4), such as in vitro supplementation with irisin (which increases the expression of PGC-1α)[33] or hydrogen sulfide (which attenuates hyperglycemia-induced formation of reactive oxygen species),[34] have been reported to effectively switch the cellular metabolism from glycolysis to OXPHOS.
Also the addition of L-ascorbic acid 2-phosphate in rabbit renal proximal tubular cells (RPTC), inhibited glycolysis and promoted OXPHOS by stimulating pyruvate utilization in mitochondria.[14] An alternative, molecular approach is based on the premise that autocrine production of TGF-β1 is responsible for the stimulation of glycolysis in long-term cultures of RPTC.[35] Indeed, RPTC treated with anti-TGF-β antibodies exhibited decreased glycolytic dominance.[35]

In contrast to more general approaches to improve oxidative metabolism (i.e. adaptations in medium substrates, or supplementation of specific compounds), an alternate, more holistic strategy was proposed by Felder et al..[36] The authors argued that factors as cell proliferation rate,[37-39] culture medium substrate composition,[40,41] and hypoxic conditions of the culture method itself[40,42] might trigger the increased glycolytic phenotype in the renal epithelial LLC-PK1 cell line. On these grounds, the authors developed a cell culture device (EpiFlow) in which continuous nutrient supply is combined with continuous oxygenation. It was reported that this device resulted in a more physiologic metabolism.[36]

Table 3. Summary of studies aimed at circumventing the Crabtree effect with mitochondrial interventions. *CCCP, carbonyl cyanide-p-chlorophenylhydrazone; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum.*

	Cells	Initial medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
Mitochondrial interventions	Rat lung cells	Primary isolated cells in Krebs-Ringer bicarbonate buffer.	**0.25mM dinitrophenol**	Incubated	Not specified	○ "The addition of 5mM glucose decreased respiration rates approximately 20%, suggesting the presence of a Crabtree effect." ○ "The classical Crabtree effect, glucose-induced depression of oxygen utilization was prevented by 0.25mM **dinitrophenol**."	Ayuso et al. 1973 (30)
	Rat heart muscle cells	Isolated heart cells incubated in Krebs-Henseleit saline (5mM glucose) and 6mU/ml insulin.	**0.05mM 2,4-dinitrophenol**	Incubated	Not specified	○ "Exogenous glucose (plus insulin) decreased oxygen consumption by the cells, providing evidence for the Crabtree effect." ○ "Oxygen consumption and glucose utilization by the cells were increased greatly by the uncoupler of oxidative phosphorylation, **2,4-dinitrophenol**."	Farmer et al. 1977 (31)
	Rat lung cells	Primary isolated cells* in Krebs-Ringer bicarbonate buffer.	**0.25mM dinitrophenol**, Krebs-Ringer bicarbonate buffer, defatted bovine serum albumin.	Incubated	± 5 minutes	○ "Uncoupling of respiration from oxidative phosphorylation by **dinitrophenol**, in agreement with previous studies on neoplastic tissues, released the inhibitory effect of glucose on lung cells respiration."	Ayuso-Parrilla et al. 1978 (32) Methods: Pérez-Diaz et al. 1977 (57)
	Rat proximal tubular epithelial cells	DMEM/F-12 (17.5mM glucose), $NaHCO_3$, 5% FBS.	HBSS, 5% FBS, glucose (conc. not specified), **4μM CCCP**	Incubated	1 day	○ "Oxidative respiration could be restored by uncoupling mitochondria with **CCCP** or by inhibition of glycolysis with **iodoacetic acid** and **2-deoxyglucose** which indicates that respiration is inhibited by glycolysis (also known as the Crabtree effect)."	Chi et al. 1995 (28) *(Also in Table 2)*

Table 4. Summary of studies aimed at circumventing the Crabtree effect with miscellaneous interventions. AscP, L-ascorbic acid 2-phosphate; ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GLM, glucose medium ; GFAM, glucose fatty acids medium; HIF1α, hypoxia-inducible factor 1α; hPSC-CMs, human pluripotent stem cell-derived cardiomyocytes; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; RPTC, renal proximal tubular cells; TCA, tricarboxylic acid cycle; TGF β, transforming growth factor beta.

	Cells	Initial medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
Miscellaneous interventions	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, **5mM glucose**, 0.44mM L-alanine, 5mM lactate, 15mM $NaHCO_3$	**25-1,000 μM L-ascorbic acid 2-phosphate (AscP)**	Incubated	6-12 days	○ "**AscP** reduced glycolysis, increased net lactate consumption by 38%, and stimulated net glucose production by 47%." ○ "Basal O2 consumption increased by 39% in RPTC grown in the presence of **AscP** and was equivalent to that in freshly isolated proximal tubules." ○ "Supplementation of media with **AscP** further improves RPTC culture conditions by promotion of cellular growth and stimulation of in vivo-like respiration, lactate utilization and net glucose synthesis."	Nowak et al. 1996 (14) *(Also in Table 1)*
	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, 5mM glucose, 0.44mM L-alanine, 2mM glycine, 5mM lactate, 15mM $NaHCO_3$, (**5 μg/ml anti-TGF-β antibodies**).	- **5 μg/ml anti-TGF-β antibodies**	Continued Incubated	12 days 6 days	○ "**TGF-β1** stimulates glycolysis, decreases respiration and, at higher concentrations, induces RPTC apoptosis and phenotypic changes." ○ "Glycolysis was not stimulated in RPTC grown in the presence of **anti-TGF-β antibodies**." ○ "RPTC treated with **anti-TGF-β antibodies** exhibited decreased glycolysis, and lactate metabolism shifted from net production to net consumption."	Nowak et al. 1996 (35)
	$LLC\text{-}PK_1$ cells	DMEM (5mM glucose), 2mM L-glutamine, 2mMpyruvate, 30μM phenol red.	**EpiFlow** cell culture perfusion device: medium perfusion rate of 2ml/hour and air delivery rate of 5-10ml/min.	Incubated	5 days	○ "**EpiFlow** maintained cells exhibited an improved oxidative metabolism as evidenced by 1) a decreased activity of glycolytic enzymes, 2) an increase in the activity of mitochondrial phosphate-dependent-glutaminase, 3) an increase in cellular ATP content, and 4) an improved morphology."	Felder et al. 2002 (36)
	bEnd3 microvascular endothelial cells	DMEM (5.5mM glucose), 2mM glutamine, 1% nonessential amino acids, 10% FBS.	DMEM (5mM or 40mM glucose), **0-300μM H_2S**	Incubated	7 days	○ "In vitro hyperglycemia resulted in a switch from OXPHOS to glycolysis, an effect that was partially corrected by **H_2S supplementation**." ○ Treatment of the cells with **H_2S** resulted in an improvement of mitochondrial respiration, whereas the hyperglycemia-induced increase in the glycolytic activity of the cells was normalized."	Suzuki et al. 2011 (34)

	Cells	Initial medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
Miscellaneous interventions	Murine C2C 12 myocytes	DMEM (25mM glucose), 10% heat inactivated FBS.	**0.78 – 50nM irisin**	Incubated	1 day	○ "Basal glycolytic metabolism was reduced following treatment with **irisin** at 2.5, 5.0 or 10.0nM for 24 hours." ○ "**Irisin treatment** for 24 hours at either 2.5, 5.0 or 10.0nM significantly elevated basal oxidative metabolism by 31.4±3.5, 14.8±0.5 and 8.5±2.3%, respectively." ○ "The oxidative reliance (represented by the ratio of oxidative metabolism to glycolytic metabolism) was significantly increased in all 24h-treated doses." ○ "**Irisin** significantly elevated metabolic gene expression including PGC-1α, NRF1, TFAM, GLUT4 and UCP3 leading to increased mitochondrial biogenesis."	Vaughan et al. 2014 (33)
	Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs)	Glucose medium (GLM) or Glucose fatty acids medium (GFAM). *See table 1.*	**Small molecule inhibition of HIF1α** with CTM, or **small molecule inhibition of LDHA** with GSKA (in GLM or GFAM cultured CMs) **siRNA inhibition of HIF1α** in GFAM cultured CMs	Incubated Incubated	7 days 96 hours	○ "**Small molecule** or **siRNA inhibition of HIF1α** or **small molecule inhibition of LDHA** results in a metabolic switch from aerobic glycolysis to oxidative phosphorylation, a more mature metabolic phenotype."	Hu et al. 2018 (25) *(Also in Table 1)*

A final, molecular approach to promote a switch from glycolysis-dominated to OXPHOS-dominated catabolism was described by Hu et al..[25] The authors observed that cardiomyocytes in the presence of glucose (concentration not specified) upregulate hypoxia-inducible factor 1α (HIF1α) and its downstream target LDH-A. Small interference RNA inhibition of HIF1α, as well as small molecule inhibition of HIF1α or LDH-A effectively restored physiologic dependence on OXPHOS (Figure 2).[25]

Discussion

For decades, cell culture is still a widely used technique, and will also serve as an important technique in the future to study the wide-ranging processes of metabolism. However, the Crabtree phenomenon that arises in cultured cells fundamentally interferes in these studies (i.e. studies focusing on susceptibility of mitochondrial toxicants,[10,43] mitochondria-related processes,[9] metabolic flexibility[1,2] and mitochondrial diseases).[44] If not taken into account, or even not recognized, this may lead to erroneous interpretations regarding mitochondrial susceptibility (reduced sensitivity to mitochondrial toxicants), the detection of non-existent metabolic shifts, and inappropriate clinical implications. Thus, in order to restore OXPHOS dominance for ATP synthesis, this study provides a comprehensive overview of reported strategies that circumvent the Crabtree effect.

The results of this review show that circumvention of the Crabtree effect–i.e. reverting the cellular metabolism from glycolysis to OXPHOS for ATP generation–is possible. Identified strategies are roughly based on suppression of glycolysis or alternatively on metabolic reprogramming. Suppression of glycolysis is either achieved by direct (e.g. use of glycolytic inhibitors) or indirect (e.g. use of galactose medium) inhibition of the glycolytic pathway. As a consequence, cells are forced to rely on other substrates than glucose that rely on OXPHOS for ATP generation. Alternatively, metabolic reprogramming aims to reprogram cells without excluding the glycolytic pathway. Reported strategies include small molecule or siRNA inhibition of HIF1α, and treatment with anti-TGF-β antibodies. While the large majority of identified reports are based on suppression of glycolysis and substrate modulation, this strategy interferes with the physiologic metabolic flexibility of mammalian cells.

So far, the mechanism of the Crabtree effect by which glucose inhibits OXPHOS remains unexplained and is probably multifactorial.[10,45,46] A widely accepted hypothesis is that glycolytic enzymes (i.e. phosphoglycerate kinase and pyruvate kinase) in the cytosol and ATP synthase compete for the phosphorylation of the available ADP pool.[47-49] According to this competition theory, the high glycolytic flux would result in low cytosolic ADP levels. As a result, the exchange of cytosolic ADP for mitochondrial ATP

across the inner mitochondrial membrane diminishes and regulatory feedback loops (i.e. pyruvate dehydrogenase inhibition by mitochondrial ATP) will be activated. Despite its attractiveness, it is important to point out that in vivo the Michaelis constant (Km) for adenine nucleotide translocator (ANT)–which facilitates the exchange of ADP and ATP across the mitochondrial inner membrane–is approximately 100 times lower than that of glycolytic enzymes.[48,49] This implies that even under rather extreme conditions, in which the activity of glycolytic enzymes increases, the cytosolic ADP would preferably be transported into the mitochondria.[48,49]

A further aspect to take into account is that the high glycolytic rates observed in cultured cells reflect the high proliferation rate of cell lines. Glycolytic switching is part of the physiological responses that accompany cell division processes in order to cope with the enhanced energy requirements and to deliver glycolytic intermediates as building blocks for the proliferating cells.[50] However, if glycolysis increases to meet the metabolic requirements for cellular growth and division, one may ask why pyruvate is converted to lactate, and not transported into the mitochondria for more ATP production. This may be explained by the fact that the high glycolytic flux of proliferating cells exceeds the maximum pyruvate dehydrogenase activity.[50,51] Another explanation might be that the conversion of pyruvate to lactate regenerates NAD^+, which is necessary for maintenance of the redox balance and continued glycolytic flux. [50] Although NADH is also converted into NAD^+ by mitochondrial complex I, this process is kinetically much slower than the conversion from pyruvate to lactate by LDH. [50]

Activation of transcription factor HIF1α–induced by hypoxic conditions in the cell culture medium–is another proposed aspect that may contribute to the high glycolytic rate in cultured cells. Yet, while shaking culture dishes – to provide an aerobic environment – partially reduces the induction of glycolysis,[13,52] oxygen tensions in standard culture conditions (140 mmHg) are found to be significantly higher than those in tissues (e.g. kidney 15-70 mmHg, brain 21-47 mmHg and uterus 15-19 mmHg).[53] Even in lung alveoli, which have the highest partial pressure of oxygen in the body, oxygen tensions are lower than in standard culture conditions (±110 mmHg versus 140 mmHg).[53] However, as static culture may come with large diffusion gradients–depending on cellular oxygen consumption rates, cell density, medium thickness and barometric pressure–significantly lower oxygen tensions may be present at the cellular level.[54-56]

Overall, the Crabtree effect can be considered a complex, and probably multifactorial phenomenon involving both external and internal factors. This is also reflected by the wide-ranging reported strategies aimed at circumventing the Crabtree effect. A first series of strategies focuses on the adjustment of external conditions.

The most widely reported 'external' strategy to circumvent the Crabtree effect, is replacement of glucose for galactose in the cell culture medium. Phosphorylation of galactose is depending on galactokinase, which is less active and can have an 8-fold slower metabolic rate than hexokinase, the first enzyme in the phosphorylation of glucose.[21,57] Consequently, as the glycolytic rate with the use of galactose decreases, cells have to rely on alternative routes (e.g. fatty acids or glutamine- driven oxidative metabolism) to maintain adequate ATP levels for cellular survival. Importantly however, although galactose is the most widely reported, and a relatively simple strategy to circumvent the Crabtree effect, it should be stressed that galactokinase kinetics differ among cell types, and some cells are even unable to metabolize galactose at all.[9,58,59] As such, the use of galactose to achieve mitochondrial dependency can be inconsistent among different cell types. Irrespective of this concern, one should realize that the use of galactose is in fact an indirect means of reducing the cellular glucose flux.

A more direct means of modulating the cellular glucose flux was evaluated by culturing cells in physiologic or low glucose conditions. The Crabtree effect remains clearly present in cells cultured at physiological levels of glucose. And although the passage of glucose through the cell-membrane is often the most rate-limiting step,[60] it might not be surprising that the Crabtree effect persists at physiological glucose concentrations as the affinity of glucose for GLUTs (with exception of GLUT2, Km ±20mM) is relatively high (GLUT1, Km ±2mM; GLUT3, Km 1-2mM; GLUT4, Km ±5mM).[61] Few studies identified a metabolic switch from glycolysis towards OXPHOS in hypoglycemic culture conditions. However, this presumably reflects a state after which all the available glucose is consumed, and cells subsequently consume other substrates that recruit OXPHOS for ATP generation. This notion is supported by Mot et al. and Arend et al. who state that cells in hypoglycemic conditions prefer glycolysis above OXPHOS until glucose is no longer available.[9,18] Although the Km for glucose for hexokinase is only 0.05mM,[60] the affinity of glucose for the glucose transporters can be assumed to be the limiting factor. In this light, it could be argued that achieving a Crabtree circumventing state requires almost total depletion of glucose from the cell culture medium.

Total depletion of glucose from the cell culture medium requires the inclusion of alternative metabolic fuel sources, and reported alternatives include the use of pyruvate, lactate, glutamine or fatty acids as single metabolic fuels. While this can be considered a feasible way of circumventing the Crabtree effect, it should be stressed that this strategy leads to complete elimination of the glycolytic pathway, and thus interferes with the metabolic flexibility of mammalian cells. This may explain the observed cessation in cell proliferation and differentiation when cells are cultured in the absence of glucose.[9,27]

An alternative strategy of regulating the glycolytic flux is with use of glycolytic inhibitors. In the context of the Warburg phenomenon many glycolytic inhibitors, targeting various levels of the glycolytic pathway, have been evaluated. So far, the variety of glycolytic inhibitors evaluated in the context of Crabtree is limited, and well-known inhibitors such as 3-bromopyruvate and 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) are not yet studied. Although these inhibitors would also be effective in limiting the glycolytic flux, one should realize that it results in metabolic inflexibility.

In summary, circumventing the Crabtree effect is possible by adjustment of external culture conditions. Alternatively, few studies focused on internal genetic reprogramming as a strategy to circumvent the Crabtree effect (i.e. inhibition of HIF1α, anti-TGF-β-antibodies, H2S and irisin). Although these strategies are promising, as it internally changes the setpoints but at the same time preserves the cellular metabolic flexibility, interventions are possible cell specific, reports are heterogenous and external validation is missing.

Conclusions

In conclusion, the Crabtree effect is a major hurdle in cell based, metabolic studies. Circumvention of the Crabtree phenomenon is possible and can be achieved by several strategies. The choice of strategy, however, should be dictated on the scientific question. For research focusing on mitochondria, it is feasible to meet mitochondrial dependence by substrate modulation in which cells are forced to rely on OXPHOS as a result of direct or indirect inhibition of glycolysis. However, the need for persistent inhibition of glycolysis interferes with restoration of normal metabolic flexibility, and would therefore severely impact studies focusing on metabolic flexibility. In this respect, it would be more preferable to use galactose-medium or metabolic reprogramming strategies.

Supplemental data

Materials and methods

The search strategy combined two complementary strategies: a systematic literature search and a citation search.

Systematic literature search

The total number of references identified in PubMed, EMBASE and Web of Science was 481, 210 and 181 respectively.

Literature search:

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Citation search

According to Web of Science 'Crabtree HG. Observations on the carbohydrate metabolism of tumours. Biochem J. 1929; 23(3): 536–545' has been cited 610 times from inception to June 2nd, 2020. Of these 610 articles, 378 focused on cultured cells.

Search strategy for Crabtree-citing articles that specifically focus on cultured cells:

ts=("Cell Culture" OR "cultured cells" OR "cultured cell" OR cultured cells OR "Cell Culture Technique" OR "cell culture" OR cell cultur* OR "tissue culture" OR "culture medium" OR "Cell Line" OR "3T3 Cells" OR "BALB 3T3 Cells" OR "NIH 3T3 Cells" OR "Swiss 3T3 Cells" OR "3T3-L1 Cells" OR "Cell Lines" OR "COS Cells" OR "HEK293 Cells" OR "RAW 264.7 Cells" OR "A549 Cells" OR "Caco-2 Cells" OR "HCT116 Cells" OR "HeLa Cells" OR "KB Cells" OR "HL-60 Cells" OR "HT29 Cells" OR "Jurkat Cells" OR "K562 Cells" OR*

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Acknowledgements

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PART III

Chapter 8

Summary, conclusions and future perspectives

Michèle J.C. de Kok

Summary

Kidney transplantation is one of the most successful medical achievements of the 20th century and has evolved as the treatment of choice in most patients with end-stage renal disease. Although a large part of the demand for donor kidneys is currently covered by grafts retrieved from living donors to compensate the lack of suitable deceased donor organs, a persistent organ shortage remains. As deceased donation brain death (DBD) donors did not suffice, the transplant community turned towards organs donated after circulatory death (DCD) as a potentially effective means to address the severe organ shortage.[1,2] Yet, from a clinical point of view, there are several non-immunological outcomes that have resulted in a reluctant attitude towards the use of these donor organs. Much of this attitude in kidney transplantation is driven by the fear of two short-term complications: early graft loss (EGL) and delayed graft function (DGF) (defined as the situation in which the recipient is temporarily dialysis dependent after transplantation). The focus of this thesis was on the underlying mechanisms and consequences of these two early complications. To obtain better insight in the impact of EGL and DGF on clinical outcomes following deceased donor kidney transplantation, we first performed a series of epidemiological evaluations that are summarized in part I of this thesis. In the second part, we focused on DGF as the primary non-immunological complication of DCD kidney transplantation and as a clinical manifestation of ischemia-reperfusion (I/R) injury. Whilst I/R injury has been studied for over 50 years, there remains a persistent challenge in translating preclinical successes to clinical achievements. In the second part of this thesis, we have therefore explored a number of methodological challenges in an effort to bridge the translational gap between bench and bed.

Part I

Chapter 2 focuses on early graft loss (EGL) as one of the most feared complications after DBD, and in particular DCD kidney transplantation. In a nationwide evaluation that included 10,307 deceased-donor kidney transplantations performed in The Netherlands between 1990 and 2018, we show that the incidence of EGL is similar after a primary DBD and DCD transplant procedure (8.1% vs. 8.5%; p=0.54). To estimate the optimal balance between the donor pool size and impact of EGL (a reticent attitude toward high-risk organs contributes to increasing organ shortages, yet a permissive policy results in an unacceptable high incidence of EGL), we systematically evaluated the consequences of EGL. Results show that EGL associates with significant detrimental consequences for the individual patient that include profound short-term and long-term mortality rates, a reduced chance of relisting and re-transplantation, and for those re-transplanted an increased risk of recurrent EGL. These observations implicate that–while the development

of EGL and the associated poor outcomes are generally attributed to the use of suboptimal kidney grafts–recipient-related factors may also elicit the development of EGL. Further evaluation shows that, despite the profound impact of EGL for the individual patient, the 8.2% overall incidence only minimally affects the survival of the transplanted patients. This implies that a policy aimed at further minimizing the incidence of EGL will lead to avoidable deaths as a result of longer waiting-list times.

Notwithstanding the EGL equivalence after DBD and DCD kidney transplantation (**chapter 2**), the higher incidence of DGF after DCD kidney transplantation remains a major impediment to an increased utilization of these kidneys in clinical practice. Yet, emerging reports indicate that despite this different and much higher DGF incidence, long-term outcomes and survival of DCD procedures are equivalent to DBD procedures.[3-5] In **chapter 3** and **chapter 4** we have attempted to find an explanation for these contrasting short-term, but equivalent long-term survival outcomes of DBD and DCD kidney transplantation.

In light of the results obtained in **chapter 2**, namely that the incidence of EGL after DBD and DCD kidney transplantation is equal, we hypothesized in **chapter 3** that prevailing concerns regarding inferior DCD outcomes are often based on data from historical cohorts, with interference of time-related effects that may no longer apply in our current timeframe. In **chapter 3** we describe the results of a nationwide, time-dependent comparative analysis. The study confirms that conclusions with regard to inferior outcomes of DCD donor grafts are interfered by time-related effects (i.e. time-varying confounding and effect modification by time) and that outcomes of DCD procedures have improved over time. In fact, whilst the 1998-2008 era associates with compromised outcomes for DCD procedures when compared with DBD procedures (higher incidences of DGF and EGL, impaired 1-year renal function in patients without DGF and inferior 1- and 5-year graft survival rates), the 2008-2018 era shows outcome equivalence for both DBD and DCD procedures. This change has occurred despite the fact that currently more older and higher risk donors and more complex recipient risk profiles are involved. To date, only the incidence of DGF remains significantly higher after DCD kidney transplantation (DBD: 16.6% vs. DCD: 39.7%; $p<0.001$). Nevertheless, patient- and graft survival equivalence for DBD and DCD procedures can be demonstrated despite the higher incidence of DGF in DCD grafts.

The notable finding of **chapter 3** that DBD and DCD graft survival is not different, despite a persistent higher incidence of DGF in DCD grafts, suggests a different impact of DGF on DBD and DCD graft survival. Our epidemiological evaluation performed in **chapter 4**, confirms this hypothesis. In fact, whilst DGF severely impacts on 10-year graft survival in DBD grafts (HR 1.67; $p<0.001$), DGF does not impact on graft survival

in DCD grafts (HR 1.08; p=0.63). Further evaluation of explanations underlying this phenomenon, shows that the severe impact of DGF in DBD grafts is not caused by a more severe DGF phenotype. On the contrary, DCD grafts are hallmarked by a more severe DGF phenotype, as indicated by a prolonged need of posttransplant dialysis and inferior renal function. As a result we then hypothesized that the differential impact reflects a difference in graft 'resilience'–i.e. the ability of the graft to cope with negative environmental changes [6]–with DCD grafts being more resilient than DBD grafts. Immunohistochemical analysis confirms the presence of resilience enhancing factors in donor kidneys. In order to map putative molecular differences in organ resilience between DBD and DCD grafts, gene expression profiling was followed by Ingenuity Pathway Analysis in pre-reperfusion kidney biopsies. Pathways relatively enriched in DCD grafts were all part of established resilience networks. Downregulated pathways appeared to be dominated by pro-inflammatory signaling cascades. These findings suggest that the different impact of DGF in DBD and DCD grafts relates to donor type-specific regulation of resilience and pro-inflammatory pathways benefitting the DCD graft and its outcomes.

Although epidemiological evaluation in **part I** shows that the impact of DGF on DCD grafts may be less stringent than commonly thought, DGF remains–albeit less prevalent– an important complication after DBD kidney transplantation. In **part II** of this thesis, we have therefore performed an evaluation of I/R injury from a more molecular perspective and how certain responses may contribute to the development of DGF.

Part II

Chapter 5 provides novel insights into the pathophysiology of DGF as clinical manifestation of I/R injury were provided. This study shows that while oxidative damage is widely considered as the key driver of I/R injury,[7] clinical observations point to a postreperfusion metabolic paralysis caused by reductive stress as the actual effector mechanism of I/R injury. To be more specific, results of a more recent work (Thesis L.G. Wijermars) has identified a fully discriminatory metabolic signature in I/R injury which is hallmarked by the inability of cells to generate high energy phosphates and a comprehensive activation of oxidation routes including glycolysis, fatty acid β-oxidation, glutaminolysis, and branched chain amino acid oxidation.[8] This compensatory catabolic state triggered by high-energy phosphate depletion results in a parallel production of reducing equivalents, i.e. NADH and $FADH_2$. Whilst these equivalents are normally oxidized through the electron transport chain (respectively complex I (NADH) and II ($FADH_2$)), or by the cytosolic LDH (glycolysis), the excess reductive load and apparent I/R-related defects in the oxidative routes may result in accumulation of reducing equivalents and therefore cause reductive stress.

Exploration of processes underlying I/R injury, critically relies on preclinical–both *in vitro* and *in vivo*–models and read-outs. Unfortunately, it appears that there is a large translational gap, as most preclinical models cannot be successfully replicated under clinical conditions. In a first attempt to bridge the translational gap, we explored methodological challenges in animal (**chapter 6**) and cell culture (**chapter 7**) studies focusing on I/R injury.

Chapter 6 focuses on potential translational hurdles between preclinical research and the clinical context. A systematic literature search identified 34 preclinical studies reporting metabolic data on I/R injury. Methodologies as well as metabolic outcomes were semi-quantified and aligned with clinical data. Results show a poor alignment of the reported preclinical metabolic data with clinical observations, and also identified a series of critical methodological shortcomings in preclinical studies (e.g. experimental set-ups that do not allow discrimination between ischemic and I/R injury, inappropriate sampling techniques, and vast diversity in the reported metabolic profiles) each compromising the interpretation and translatability of the preclinical studies.

Chapter 7 elaborates on mammalian cell culture as another promising model to study the processes of I/R injury and it focuses on the Crabtree effect[9] which emerges as a major challenge in this field of research. The Crabtree effect refers to the adaptation of cultured cells to a glycolytic phenotype, away from oxidative phosphorylation. By conducting a systematic literature search we showed that circumventing the Crabtree effect (i.e. reverting the metabolism of cultured cells from glycolysis back to oxidative phosphorylation for adenosine triphosphate generation) is possible. Strategies are roughly based on the suppression of glycolysis (either direct by the use of glycolytic inhibitors, or indirect by the use of galactose medium), or alternatively on metabolic reprogramming (e.g. inhibition of HIF1α, anti-TGF-β-antibodies, H2S and irisin). The choice of strategy, however, should be dictated by the scientific question.

Conclusions

The first part of this thesis demonstrates that kidneys from DCD donors can be fully embraced, and that the perception of DCD kidney transplantation being inferior to DBD kidney transplantation – that is mainly motivated by concerns regarding high incidences of DGF and EGL – is no longer justified. One key explanation for this phenomenon is that outcomes of transplanted DCD kidneys have improved over time, with a similar incidence of EGL following DBD and DCD kidney transplantation in the current era. A further explanation is that both donor types have a different biological response to DGF: whilst DGF severely impacts on DBD graft survival, DGF has no impact on DCD graft

survival. Our molecular evaluation performed suggests that this different impact relates to donor type-specific regulation of resilience and pro-inflammatory pathways benefitting the DCD graft and its outcomes. As such, the persistent high incidence of DGF in DCD grafts should not be regarded an impediment toward the use of these donor kidneys.
The second part of this thesis identifies and explores a series of physiological (i.e. the Crabtree effect) and methodological (i.e. experimental set-ups) contrasts between preclinical I/R injury models and the clinical reality, that all may contribute to the impaired translatability of preclinical studies in clinical practice. Awareness of these challenges and pitfalls may help to improve study designs in future research, bringing us one step closer towards bridging the translational gap.

Future perspectives

As in most studies, our attempt to answer a few questions has lead to new challenges and many more questions.

One notable aspect that emerged from this thesis, is that–although the development of early and late graft loss is generally attributed to the use of suboptimal kidney grafts – the actual impact of donor characteristics on graft survival is less profound than assumed. This is not only supported by the paradoxical finding that transplant outcomes improve whilst we accept many more older and higher risk donor organs, but also by the remarkably poor association between (i) the kidney donor risk index (KDRI) and 5-year graft survival, and (ii) traditional donor/procedural risk factors and EGL (chapter 2, 3). Hence, these data suggest that we can focus less on donor characteristics. The importance of recipient factors, however, is underpinned from the observation that EGL patients have a reduced chance of relisting (for re-transplantation), and an increased risk of recurrent EGL when re-transplanted. From this perspective, it would be of interest to shift future research from a primarily *donor* focused towards a more *recipient* or *donor-in-host* focused research. This might help us to better identify recipient factors, and donor-recipient interactions that impact on transplant outcomes.

Since our results show that the incidence of EGL has been decreasing over time, we may conclude that we are heading in the right direction. An intriguing and still underexposed question, however, is whether this intuitive response is justified. A policy aimed at (further) minimizing the incidence of EGL could lead to 'cherry-picking' of organs, which would contribute to the persistent organ shortage and prolong time on the waiting list. Therefore, although *a risk-avoiding* policy may be natural among doctors facing their patients (not taking unnecessary risks for the safety of the patient), it is debatable

whether it would be more justified to strive for a *risk-accepting* policy for the 'hidden' patients on the waiting list.

Another fascinating observation in the first part of this thesis is that although DGF is often considered a relative contra-indication for the use of DCD kidneys, DGF does not impact on graft survival in DCD grafts (whereas it does negatively impact on graft survival in DBD grafts). We hypothesized that this differential impact reflects differences in graft 'resilience' (i.e. the ability of the graft to cope with negative environmental changes [6]). Indeed, molecular evaluation showed relative enrichment of resilience-associated pathways in DCD versus DBD grafts. Although these observations are only based on donor kidneys, it is of interest to test whether this observation for the kidney also applies to other organs and what molecular signals underlie the selective activation of these pathways. This can subsequently serve as a basis to investigate whether organ resilience can be modulated, which could be beneficial in terms of organ preservation and preconditioning in a broad variety of major surgical interventions that induce ischemia and reperfusion (e.g. in transplantation, cardiothoracic and vascular surgery).

In the second part of this thesis we have explored challenges and methodological shortcomings that arise in *in vitro* and *in vivo* studies focusing on I/R injury. For cell culture studies, the Crabtree effect must be considered when designing future experiments. For preclinical studies, the first step was taken to identify pitfalls and provide recommendations for future research models. Integrating this theory into research practice may be an important next step towards the ultimate goal to narrow the translational gap between bench and bed, and achieve a successful targeted intervention that will benefit our patients.

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Chapter 9

Nederlandse samenvatting

Michèle J.C. de Kok

Samenvatting

Voor patiënten met eindstadium nierfalen is een nierfunctievervangende behandeling een voorwaarde voor overleving die bestaat uit hemodialyse, peritoneaal dialyse of een niertransplantatie. Alhoewel de keuze van nierfunctievervangende therapie kan wisselen per patiënt, heeft niertransplantatie over het algemeen de voorkeur vanwege voordelen op het gebied van overleving, kwaliteit van leven en zorgkosten. [1-3]

Een van de eerste succesvolle niertransplantaties in mensen werd uitgevoerd in 1954 in Boston, tussen tweelingbroers.[4] Sindsdien zijn er vele transplantaties uitgevoerd met nieren van zowel levende als hersendode donoren (*Donation after Brain Death*, ook wel DBD).[5] Echter, hebben zowel het medische succes van de niertransplantatie, als ook de toenemende patiëntpopulatie met nierfalen (als gevolg van vergrijzing en een hogere prevalentie van diabetes en hypertensie), geleid tot een aanzienlijke mismatch tussen vraag en aanbod van niertransplantaties.[6,7] Dit wordt weerspiegeld door lange wachtlijsten en heeft geleid tot het overlijden van veel patiënten tijdens het wachten op een niertransplantatie.

In een poging de wachtlijst te verkleinen, heeft de transplantatiegemeenschap zich gewend tot nieren die worden gedoneerd na een circulatiestilstand (*Donation after Circulatory Death*, ofwel DCD) om het aanbod van donornieren te vergroten.[5,8] Bij een DBD donor wordt het overlijden vastgesteld op grond van neurologische criteria, waarmee de procedure verschilt met een DCD donor, waarbij het overlijden wordt vastgesteld op basis van circulatie criteria.[9] Als gevolg hiervan worden nieren van DCD donoren blootgesteld aan een onvermijdelijke periode van warme ischemie voorafgaande aan operatie (Figuur 1, hoofdstuk 1).

Ondanks het feit dat nieren van DCD donoren het tekort aan donororganen kan verminderen, blijven veel landen zeer terughoudend met het transplanteren van deze nieren.[7,10] Hoewel deze terughoudendheid voor sommige landen is gebaseerd op wettelijke beperkingen, ethische kwesties of logistieke uitdagingen,[7] komt het in de meeste landen door zorgen om een hoge incidentie op *vroeg transplantaat falen* en *delayed graft function (*DGF; het vertraagd op gang komen van de nier na transplantatie, en een klinische manifestatie van ischemie-reperfusie [I/R] schade).[10-12] Hierin is het vroegtijdig falen van een niertransplantaat een vanzelfsprekende ernstige complicatie, en is er bezorgdheid over DGF aangezien dit in verband wordt gebracht met een verminderde nierfunctie en verminderde transplantaatoverleving op de lange termijn.[13-14]

Het eerste deel van dit proefschrift concentreert zich op de vraag of de algemene perceptie dat DCD niertransplantatie inferieur is aan DBD niertransplantatie nog steeds correct is. Het tweede deel van dit proefschrift gaat verder in op DGF als primaire complicatie van DCD niertransplantatie en als uiting van I/R schade. Hoewel I/R schade al meer dan 50 jaar wordt bestudeerd, blijft het vertalen van preklinische (*in vitro* en *in vivo*) successen naar de klinische setting beperkt. In het tweede deel van dit proefschrift worden daarom een aantal methodologische en fysiologische uitdagingen onderzocht in een poging deze translationele kloof te overbruggen.

Deel I

In **hoofdstuk 2** focussen we op *vroeg transplantaat falen* (binnen 90 dagen na transplantatie) als een van de meest gevreesde complicaties na DBD en in het bijzonder DCD niertransplantatie. In een nationale evaluatie waarin 10.307 postmortale niertransplantaties zijn geïncludeerd (uitgevoerd in Nederland tussen 1990 en 2018), laten we zien dat de incidentie van vroeg transplantaat falen gelijk is na een primaire DBD en DCD procedure (8,1% vs. 8,5%; p=0,54). Vervolgens evalueerden we de consequenties van vroeg transplantaat falen om de optimale balans tussen de grootte van de donorpool en de impact van vroeg transplantaat falen in te schatten. Immers, waar een terughoudend beleid ten aanzien van risicovolle organen resulteert in toenemende orgaantekorten, resulteert een tolerant beleid in het accepteren van deze organen in een onaanvaardbaar hoge incidentie van vroeg transplantaat falen. De resultaten laten zien dat vroeg transplantaat falen gepaard gaat met significant nadelige gevolgen voor de individuele patiënt: patiënten met vroeg transplantaat falen hebben een significant hogere kans op overlijden op de korte en lange termijn, een kleine kans op een re-transplantatie en, wanneer de patiënt wel voor re-transplantatie in aanmerking komt, een verhoogde kans op recidief vroeg transplantaat falen. Terwijl vroeg transplantaat falen vaak wordt geweten aan het gebruik van suboptimale donornieren, impliceren deze bevindingen dat juist ook ontvanger-specifieke factoren een belangrijke rol spelen in de ontwikkeling van vroeg transplantaat falen. Verdere analyse toont aan dat, ondanks de grote impact van vroeg transplantaat falen op de individuele patiënt, de totale incidentie van 8,2% de overleving van het gehele cohort getransplanteerde patiënten slechts minimaal beïnvloedt. Dit impliceert dat een beleid gericht op het verder verlagen van de incidentie van vroeg transplantaat falen zal leiden tot een toename in vermijdbare sterfgevallen op de wachtlijst als gevolg van langere wachttijden voor transplantatie.

Alhoewel de incidentie van vroeg transplantaat falen gelijk is na DBD en DCD niertransplantatie (**hoofdstuk 2**), blijft de hogere incidentie van DGF na DCD niertransplantatie een belangrijke drempel voor het accepteren en transplanteren van DCD donornieren. Hier staat tegenover dat recente onderzoeken laten zien dat, ondanks de hogere incidentie van DGF na een DCD niertransplantatie ten opzichte van

DBD niertransplantatie, de lange termijn uitkomsten en overleving gelijk zijn in beide groepen.[10,15-16] In **hoofdstuk 3 en 4** hebben we geprobeerd een verklaring te vinden voor deze contrasterende korte termijn (verschil in DGF incidentie), maar gelijke uitkomsten op de lange termijn na DBD en DCD niertransplantatie.

In het licht van de resultaten verkregen in **hoofdstuk 2**, namelijk dat de incidentie van vroeg transplantaat falen gelijk is na DBD en DCD niertransplantatie, veronderstelden we dat de heersende bezorgdheid met betrekking tot inferieure DCD uitkomsten vaak gebaseerd is op data van historische cohorten, waarbij de uitkomsten–door tijd gerelateerde effecten – mogelijk niet meer van toepassing zijn op het huidige tijdsbestek. In **hoofdstuk 3** beschrijven we de resultaten van een landelijke *time-dependent comparative* analyse. Deze studie bevestigt dat conclusies met betrekking tot inferieure uitkomsten van DCD niertransplantaten, worden beïnvloed door tijd gerelateerde effecten (tijd-variërende confounding en effect modificatie door tijd) en dat de uitkomsten van DCD procedures zijn verbeterd over tijd. Waar tussen 1998 en 2008 de uitkomsten na DCD niertransplantatie inferieur waren ten opzichte van DBD transplantatie (hogere incidentie van DGF en vroeg transplantaat falen, verminderde nierfunctie na 1 jaar bij patiënten zonder DGF, en verminderde transplantaatoverleving na 1 en 5 jaar), zijn tussen 2008 en 2018 de uitkomsten gelijk (met uitzondering van een hogere incidentie DGF na DCD niertransplantatie). Deze verschuiving deed zich voor ondanks het feit dat er in het meest recente tijdvak meer transplantaties werden uitgevoerd bij oudere ontvangers, met nieren van oudere en hoog risico donoren. Zoals eerdergenoemd blijft de incidentie van DGF significant hoger na een DCD niertransplantatie (DBD: 16,6% versus DCD: 39,7%; p<0,001). Echter, ondanks de hogere incidentie, laten resultaten zien dat zowel de patiënt- als transplantaatoverleving gelijk is na DBD en DCD niertransplantatie.

De opmerkelijke bevinding van **hoofdstuk 3** dat DBD en DCD transplantaatoverleving gelijk is ondanks een hogere incidentie van DGF in DCD transplantaten suggereert dat er een verschillende impact is van DGF op de overleving van DBD en DCD transplantaten. Onze epidemiologische analyse uitgevoerd in **hoofdstuk 4** bevestigt deze hypothese: terwijl DGF een zware impact heeft op de 10-jaars transplantaatoverleving van DBD transplantaten (HR 1,67; p<0,001), heeft DGF geen invloed op de transplantaatoverleving van DCD transplantaten (HR 1,08; p=0,63). Nadere analyse naar een verklaring die ten grondslag ligt aan dit fenomeen laat zien dat de grote impact van DGF in DBD transplantaten niet wordt veroorzaakt door een ernstiger DGF-fenotype. Integendeel, de mate van DGF was ernstiger in DCD transplantaten met een langere behoefte aan dialyse na transplantatie en een verminderde nierfunctie. Een andere verklaring voor de verschillende impact van DGF in DBD en DCD niertransplantatie is een verschil in *veerkracht* van het transplantaat (d.w.z. het vermogen van het transplantaat om met negatieve veranderingen om te gaan)[17] waarbij nieren van DCD donoren veerkrachtiger

zijn dan nieren van DBD donoren. Immunohistochemische analyse bevestigde de aanwezigheid van veerkracht verhogende factoren in donornieren. In een poging een verschil aan te tonen in veerkracht tussen DBD en DCD nieren, hebben we gekeken naar genexpressie en een *Ingenuity Pathway Analysis* uitgevoerd in pre-reperfusie nierbiopten. Pathways die relatief verrijkt waren in DCD nieren maken allen deel uit van gevestigde veerkracht netwerken. De relatief gedownreguleerde pathways in DCD nieren worden gedomineerd door pro-inflammatoire signaalcascades. Deze bevindingen suggereren dat het verschil in impact van DGF in DBD en DCD nieren verband houdt met donortype specifieke regulatie van veerkracht en pro-inflammatoire cascades die de DCD transplantaten en klinische uitkomsten ten goede komt.

Hoewel de epidemiologische evaluatie uit **deel I** laat zien dat de impact van DGF op DCD minder groot is dan in het algemeen wordt aangenomen, blijft DGF – zij het minder vaak voorkomend – een belangrijke complicatie na DBD niertransplantatie. In **deel II** van dit proefschrift, hebben we derhalve een evaluatie uitgevoerd naar I/R schade vanuit een meer moleculair perspectief.

Deel II

Hoofdstuk 5 biedt nieuwe inzichten in de pathofysiologie van DGF als een klinische manifestatie van I/R schade. Deze studie laat zien dat hoewel oxidatieve stress vaak wordt beschouwd als meest belangrijke veroorzaker van I/R schade,[18] er in de klinische setting juist sprake lijkt te zijn van een post-reperfusie metabole verlamming ten gevolge van reductieve stress. Meer specifiek laat eerder onderzoek (Thesis L.G. Wijermars) zien dat I/R schade wordt gekenmerkt door een totale metabole uitputting waarbij de mitochondriën niet in staat zijn voldoende energie (adenosinetrifosfaat (ATP)) te genereren. Als gevolg hiervan worden andere routes ter compensatie geactiveerd waaronder glycolyse, vrij vetzuren β-oxidatie, glutamine oxidatie en branched chain amino zuur oxidatie.[19] Deze compenserende katabole conditie resulteert in een parallelle productie van NADH en $FADH_2$. Waar oxidatie van deze equivalenten normaal plaatsvindt in de mitochondriale elektronentransportketen (respectievelijk complex I (NADH) en complex II ($FADH_2$)), of door lactaatdehydrogenase (glycolyse), leidt I/R-gerelateerde schade tot een onvermogen hiertoe, met reductieve stress door accumulatie van NADH en $FADH_2$ als resultaat.

Onderzoek naar processen die ten grondslag liggen aan I/R schade zijn veelal gebaseerd op preklinische – zowel *in vitro* als *in vivo* – modellen. Helaas blijkt er echter sprake van een grote translationele kloof, doordat preklinische prestaties niet succesvol kunnen worden vertaald naar de klinische setting. In een eerste poging deze translationele kloof te overbruggen, hebben we methodologische uitdagingen in dier- (**hoofdstuk 6**) en celkweek (**hoofdstuk 7**) studies naar I/R schade onderzocht.

In **Hoofdstuk 6** focussen we op potentiële discrepanties tussen preklinisch (in vivo) onderzoek en de klinische context. Met behulp van een systematisch literatuuronderzoek identificeerden we 35 preklinische dierstudies die metabole data rapporteerden in de context van renale I/R schade. De toegepaste methoden en metabole uitkomsten werden in kaart gebracht en vergeleken met de klinische data. Er werden meerdere methodologische tekortkomingen in preklinische studies aan het licht gebracht (o.a. het gebrek aan onderscheid tussen I/R en I/R *schade*, onjuist gebruik van sample technieken, en incomplete rapportage van metabole profielen), welke de interpretatie en vertaalbaarheid naar de klinische setting bemoeilijken. Dit werd bevestigd door de metabole data, die grote variabiliteit tussen dier en mens onthulde.

Hoofdstuk 7 gaat dieper in op celkweek als een alternatief, veelbelovend model om processen van I/R schade te bestuderen. In dit hoofdstuk focussen we op het Crabtree effect[20] dat een belemmering vormt voor studies naar mitochondriën en metabole flexibiliteit. Het Crabtree effect beschrijft het fenomeen dat cellen in standaard kweekmedium afhankelijk zijn van glycolyse, in plaats van oxidatieve fosforylering (OXPHOS) voor ATP generatie. Met behulp van een systematisch literatuuronderzoek hebben we laten zien dat het Crabtree effect omzeild kan worden waardoor cellen een meer fysiologisch metabolisme handhaven en OXPHOS-dominant in plaats van glycolyse-dominant zijn voor ATP productie. Strategieën om dit te bereiken kunnen grofweg worden onderverdeeld in de directe en indirecte onderdrukking van de glycolyse (door respectievelijk het gebruik van glycolyse remmers en door het gebruik van galactose medium), of door metabole herprogrammering (bijv. remming van HIF1α, gebruik van anti-TGF β, irisine, H2S). De keuze van strategie hangt echter sterk af van de te beantwoorden onderzoeksvraag.

Conclusie

Het eerste deel van dit proefschrift laat zien dat nieren van DCD donoren volledig kunnen worden omarmd voor transplantatie en dat de algehele perceptie dat DCD niertransplantatie inferieur is aan DBD niertransplantatie, wat veelal wordt gemotiveerd door zorgen omtrent een hoge incidentie van DGF en vroeg transplantaat falen, niet langer gerechtvaardigd is. Een belangrijke verklaring voor deze conclusie is dat de uitkomsten van DCD niertransplantaties zijn verbeterd over de tijd, met een gelijke incidentie van vroeg transplantaat falen na DBD en DCD niertransplantatie in het huidige tijdsbestek. Een bijkomende verklaring is dat beide donortypen een verschillende biologische respons hebben op DGF: terwijl DGF een ernstige impact heeft op de overleving van DBD transplantaten, heeft DGF geen invloed op de transplantaat overleving van DCD nieren. De uitgevoerde moleculaire analyse suggereert dat dit

verband houdt met donortype specifieke regulatie van veerkracht en pro-inflammatoire cascades, die de DCD transplantaten en resultaten ten goede komt. De persisterende hogere incidentie van DGF in DCD transplantaten moet dus niet worden beschouwd als een belemmering voor het accepteren van deze donornieren.

Het tweede deel van dit proefschrift exploreert multipele fysiologische (het Crabtree effect) en methodologische (experimentele opstellingen) uitdagingen in preklinische en klinische onderzoeken gericht op I/R schade, welke de vertaalbaarheid van preklinische successen naar de klinische setting bemoeilijken. Bewustwording van deze valkuilen draagt bij aan het verbeteren van toekomstige onderzoeksprotocollen, als eerste stap naar de overbrugging van deze translationele kloof.

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Appendices

Curriculum Vitae
Dankwoord

Curriculum Vitae

Michèle J.C. de Kok was born on the 11th of February 1991 in Barcelona, Spain. After living in Spain and Indonesia for a couple of years, she moved with her parents and older sister Elise to Valkenswaard, the Netherlands. Michèle graduated from the secondary school in Valkenswaard (Gymnasium, Were Di) and started Medical School at the Leiden University Medical Center in 2009.

During her studies, she joined the student society of Minerva where she participated in several committees. She joined the board of the Panacee medical committee, was the chair of Agnodice and organized the 33th edition of the Veerstichting Symposium (Feather Foundation) in 2012. Starting 2010, Michèle also worked as a chief retrieval technician at the BISLIFE Foundation. In this function, she was responsible for the surgical retrieval of post mortem heart valves and corneas meant for transplantation.

Michèle obtained her medical degree in November 2016 and started working as a surgical resident not in training (ANIOS) at the Department of Surgery at the HagaZiekenhuis, The Hague, for one year. After this, she worked as a PhD researcher at the Department of Surgery (Transplant Center) at the Leiden University Medical Center, to study ischemia/reperfusion injury in kidney transplantation. Prof. dr. I.P.J. Alwayn, dr. J.H.N Lindeman and dr. A.F.M. Schaapherder served as supervisors of this PhD program. During her time as a researcher she was offered the possibility to participate in several scientific courses, to present research on multiple (inter)national meetings, and to publish scientific papers that have led to this thesis.

From 2021, Michèle resumed her clinical work at the surgical department at the Haaglanden Medisch Centrum, The Hague, after which she was accepted into the Surgery Residency Program. As of January 1st 2022, she started her residency at the Leiden University Medical Center under supervision of dr. A. Schepers and will continue her training at the Haaglanden Medisch Centrum under supervision of dr. H.A. Leijdesdorff.

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