Transplant International

ORIGINAL ARTICLE

# Cell cycle arrest and cell death correlate with the extent of ischaemia and reperfusion injury in patients following kidney transplantation – results of an observational pilot study

Felix C. F. Schmitt[1], Eduardo Salgado[1], Janina Friebe[1], Thomas Schmoch[1], Florian Uhle[1], Thomas Fleming[2,3], Johanna Zemva[2], Lars Kihm[2], Christian Nusshag[4], Christian Morath[4], Martin Zeier[4], Thomas Bruckner[5], Arianeb Mehrabi[6], Peter P. Nawroth[2,3,7], Markus A. Weigand[1], Stefan Hofer[8] & Thorsten Brenner[1]

1 Department of Anesthesiology, Heidelberg University Hospital, Heidelberg, Germany
2 Department of Internal Medicine I and Clinical Chemistry, University Hospital Heidelberg, Heidelberg, Germany
3 German Center for Diabetes Research (DZD), Neuherberg, Germany
4 Department of Nephrology, Heidelberg University Hospital, Heidelberg, Germany
5 Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany
6 Department of General, Visceral and Transplant Surgery, Heidelberg University Hospital, Heidelberg, Germany
7 Joint Division Molecular Metabolic Control, German Cancer Research Center (DKFZ) Heidelberg Center for Molecular Biology (ZMBH) and University Hospital Heidelberg University, Heidelberg, Germany Institute for Diabetes and Cancer IDC Helmholtz Center Munich and Joint Heidelberg-IDC Translational Diabetes Program, Neuherberg, Germany
8 Department of Anesthesiology, Kaiserslautern Westpfalz Hospital, Kaiserslautern, Germany

## SUMMARY

A prolonged cold ischaemia time (CIT) is suspected to be associated with an increased ischaemia and reperfusion injury (IRI) resulting in an increased damage to the graft. In total, 91 patients were evaluated for a delayed graft function within 7 days after kidney transplantation (48 deceased, 43 living donors). Blood and urine samples were collected before, immediately after the operation, and 1, 3, 5, 7 and 10 days later. Plasma and/or urine levels of total keratin 18 (total K18), caspase-cleaved keratin 18 (cc K18), the soluble receptor for advanced glycation end products (sRAGE), tissue inhibitor of metalloproteinase-2 (TIMP-2) and insulin-like growth factor-binding protein-7 (IGFBP7) were measured. As a result of prolonged CIT and increased IRI, deceased donor transplantations were shown to suffer from a more distinct cell cycle arrest and necrotic cell death. Plasmatic total K18 and urinary TIMP-2 and IGFBP7 were therefore demonstrated to be of value for the detection of a delayed graft function (DGF), as they improved the diagnostic performance of a routinely used clinical scoring system. Plasmatic total K18 and urinary TIMP-2 and IGFBP7 measurements are potentially suitable for early identification of patients at high risk for a DGF following kidney transplantation from deceased or living donors.

*Transplant International* 2018; 31: 751–760

**Key words**
cell cycle arrest, cell death, delayed graft function, ischaemia and reperfusion injury, kidney transplantation, total keratin 18

Received: 14 May 2017; Revision requested: 26 June 2017; Accepted: 28 February 2018; Published online: 30 March 2018

**Correspondence**
Thorsten Brenner MD, Department of Anesthesiology, Heidelberg University Hospital, 110, Im Neuenheimer Feld, D-69120 Heidelberg, Germany.
Tel.: +49-6221 56-6351;
fax: +49-6221 56-5345;
e-mail: thorsten.brenner@med.uni-heidelberg.de


doi:10.1111/tri.13148

## Introduction

A large number of congenital and acquired diseases ultimately lead to terminal renal failure with the requirement of renal replacement therapy (RRT). RRT is known to result in a high level of dependency and a potential handicap for patients in their everyday lives [1]. To break this vicious circle, many patients decided to undergo kidney transplantation. The majority of kidney transplantations are carried out with organs from deceased donors (DD) [2]. In Germany, kidneys from living donors are removed and transplanted in the same hospital; therefore, living donor (LD) grafts only suffer from a short cold ischaemia time (CIT). The problem with grafts from DDs is that they are usually removed in a different hospital and then have to be transferred to the transplantation centre, which inevitably leads to a prolonged CIT. Furthermore, it is well known that prolonged CIT is one crucial factor predisposing for ischaemia and reperfusion injury (IRI) as well as delayed graft function (DGF) development [3,4]. However, other variables of organ quality such as donor age, serum creatinine, comorbities (hypertension, diabetes mellitus) and manner of death (cardiac arrest, brain death) influence the organ susceptibility for DGF and IRI essentially [5–7]. Interestingly, the degree of acute tubular necrosis (ATN) in renal transplant biopsies does not predispose for DGF [8]. In fact, rather chronic histological findings in the form of interstitial fibrosis and vascular intima thickening seem to be of major relevance [5]. In addition, the interaction of recipient and donor characteristics only explains a fraction of the risk for DGF. Significant differences in treatment protocols of transplantation centres are considered to result in varying DGF prevalences [9]. According to the literature, the number of patients with a DGF varies between 20% and 40% [7,10]. Moreover, these organs are at high risk to be irreversibly damaged, so that they will never be able to achieve a sufficient function. The impact of prolonged CIT on the clinical course, the immunoinflammatory response, as well as the related cell cycle arrest and cell death, has not been fully clarified yet. TIMP-2 and IGFBP7 are both biomarkers for cellular stress, which are involved in the G1 cell cycle arrest. The combination of both biomarkers [(TIMP-2) × (IGFBP-7)] has recently been shown to be of potential value for the prediction of a DGF in patients following deceased donor transplantation [11]. Moreover, in a small pilot study, a work group from the Netherlands reported a relation between CIT and the ensuing cell death mechanisms [12]. Depending on the type of cell death (apoptosis vs. necrosis), specific keratin 18 (K18) isoforms (caspase-cleaved vs. full-length, uncleaved K18) are released into the bloodstream. Quantification of isoform-specific epitopes therefore enables the assessment of the predominant mode of cell death in the individual patient. Thus, the aims of this study were (i) to assess the impact of CIT on the extent of IRI by the use of cell cycle arrest and cell death biomarkers and (ii) to evaluate their use for early diagnosis of a delayed graft function in patients undergoing kidney transplantation from deceased or living donors.

## Materials and methods

The observational clinical study was approved by the local ethics committee (Ethics Committee of the Medical Faculty of Heidelberg, Trial Code No. S-441/2011/German Clinical Trials Register: DRKS00003483). All study patients gave written informed consent. In total, 91 patients undergoing kidney transplantation were enrolled from January 2012 to January 2015. A total of 48 patients received a graft from deceased donors, whereas 43 underwent living donor transplantation. ABO incompatibility was defined to be an exclusion criterion. Relevant baseline data, clinical data and routine blood parameters were collected, and patients were re-evaluated for short- and long-term complications 10, 30, 90 and 180 days after the transplantation. DGF was defined as low diuresis (<1000 ml excretion/day) despite forced medical stimulation and/or need for RRT up to 7 days following kidney transplantation. Blood and urine samples were collected before transplantation (Pre), immediately after the end of the surgical procedure (d0), and 1 day (d1), 3 days (d3), 5 days (d5), 7 days (d7) and 10 days (d10) afterwards. Up to d10, we were able to collect urine samples from all LD patients (number of urine samples at d0 = 31 and at d1 = 40). In the DD group, one patient was not able to give an urine sample during the 10-day observation period due to continuous RRT requirement (number urine samples at d0 = 29 and at d1 = 36). Prior to transplantation, no urine samples could be collected in the DD group due to an absent urinary excretion in all patients. For quantitative determination of total keratin 18 (total K18) and caspase-cleaved keratin 18 (ccK18) in plasma and urine samples, enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (M65 Epideath and M30 Apoptosense: Peviva AB, Bromma, Sweden/sRAGE: R&D Systems, Minneapolis, MN, USA) were used. By the use of an antibody array (Proteome Profiler Human Kidney Biomarker Array: R&D Systems), plasmatic soluble RAGE (sRAGE) was identified to be the most suitable marker for the assessment of ongoing kidney damage. For quantitative

determination of sRAGE in plasma, ELISA kits according to the manufacturer's instructions (R&D Systems) were used. All assays were performed in duplicate. Urinary levels of TIMP-2 and IGFBP-7 were measured in a combined commercial assay [TIMP-2] × [IGFBP7] utilizing the immunoassay method integrated with the Astute 140 Meter Kit (Astute Medical Inc., San Diego, CA, USA). All values for [TIMP-2] × [IGFBP7] are reported in units of $(ng/ml)^2/1000$.

The resulting data were entered into an electronic database (Excel 2010; Microsoft Corp, Redmond, WA, USA) and evaluated using the SPSS software (Version 21.0; SPSS Inc, Chicago, IL, USA). Categorical data were summarized using absolute and relative frequencies. Quantitative data were summarized using median with quartiles. The Kolmogorov–Smirnov test was applied to check for normal distribution. Because of non-normally distributed data, nonparametric methods for evaluation were used (chi-square test for categorical data and Mann–Whitney *U* test for continuous data). Optimism-corrected, monoparametric receiver operating characteristic (ROC) analyses were computed by a fivefold cross-validation procedure. Concerning the prediction of a delayed graft function within the 10-day observation period, additional non-optimism-corrected, multiparametric ROC analyses were performed. Correlation analyses were performed by calculating the Spearman's rank correlation coefficient (Spearman's Rho/ρ). A *P*-value <0.05 was considered statistically significant.

## Results

### Patients' characteristics

A detailed overview of the patients' characteristics in the two study groups (DD: $n = 48$ vs. LD: $n = 43$) as well as postoperative graft function is described in Table 1. Patients undergoing living donor transplantation were shown to be significantly younger and less dependent on RRT prior to transplantation (DD group: 100%, LD group: 83.7%). Cold ischaemia time (CIT) was shown to be significantly prolonged in the DD group, whereas warm ischaemia time (WIT) did not differ significantly between the two study groups.

### Donor characteristics and graft function

Immediately following kidney transplantation, a satisfying graft function could be achieved in all LD patients ($n = 43$; 100%) and none of these patients required RRT up to d10 (Table 1). In patients undergoing deceased donor transplantation, a sufficient early graft function could only be observed in 29 patients (60.4%). Despite forced medical stimulation with loop diuretics, 19 patients (39.6%) had a significantly reduced diuresis alone or in combination with the requirement of an initial RRT. A subsequent long-term follow-up was able to confirm that patients of the DD group require RRT more frequently and are hallmarked by significantly reduced glomerular filtration rates as well as higher serum creatinine levels.

### Immune response

Acute-phase reactions were shown to be more pronounced in DD patients as assessed by increased plasma levels of C-reactive protein (CRP) (Table 2). Moreover, plasma levels of sRAGE were shown to be significantly increased in DD graft recipients in the early phase after transplantation with peak concentrations at d5.

### Cell cycle arrest biomarkers

As a combinational biomarker for cell cycle arrest in urine samples, [TIMP-2] × [IGFBP7] was shown to be significantly increased in the DD group in comparison with the LD group at early stages after kidney transplantation (at d0 and d1) (Fig. 1a). This difference was also true for patients suffering from a delayed graft function in comparison with those with a normal graft function (at d1 and d3) (Fig. 1b). The glomerular filtration rate (as indirectly assessed by the creatinine-based CKD-EPI – as well as MDRD-formulas) in patients suffering from a DGF was shown to be decreased throughout the whole observation period from d1 up to d10 (Figs 1c and S1).

### Cell death biomarkers

Plasma levels of total K18 were significantly elevated already before transplantation and remained increased up to d5 in DD graft recipients in comparison with LD graft recipients (Fig. 2a). Concentrations of total K18 in urine samples revealed a comparable levelling, whereas significant differences could only be obtained at d0 (Fig. 2b). Plasma levels of ccK18 did not differ significantly between the two study groups and urine concentrations of ccK18 also failed to be of informative value (Table 2).

### Prediction of a DGF in patients following kidney transplantation

(i) *Monoparametric ROC analyses*. Concerning the prediction of a DGF within the 10-day observation period,

**Table 1.** Patients' characteristics.

| | Deceased donors ($n$ = 48) | Living donors ($n$ = 43) | $P$-value |
|---|---|---|---|
| Recipient | | | |
| Age (years) | 57.0 (43.0–65.0) | 45.0 (36.0–51.0) | **<0.001** |
| Height (cm) | 171.0 (163.0–177.5) | 173.0 (164.0–178.0) | 0.299 |
| Weight (kg) | 70.0 (58.3–84.8) | 79.0 (67.0–91.0) | 0.066 |
| Dialysis prior to transplantation ($n$) | 48 (100%) | 36 (83.7%) | **<0.001** |
| Donor | | | |
| Age (years) | 57.0 (48.0–73.0) | 54.0 (48.0–60.0) | 0.110 |
| Height (cm) | 170.0 (170.0–175.5) | 170.0 (160.0–178.0) | 0.219 |
| Weight (kg) | 75.0 (70.0–77.5) | 79.0 (65.0–87.0) | 0.422 |
| Serum creatinine (mg/dl) | 0.6 (0.4–0.8) | 0.7 (0.6–0.9) | 0.175 |
| CRP (mg/l) | 102.0 (37.9–180.3) | 2.0 (2.0–2.5) | **<0.001** |
| Graft | | | |
| Cold ischaemia time (min) | 780 (560–960) | 175 (152–195) | **<0.001** |
| Warm ischaemia time (min) | 40 (30–59) | 33 (28–54) | 0.258 |
| Operation time (min) | 183 (146–238) | 145 (120–184) | **0.01** |
| Graft outcome | | | |
| Immediate graft function* | 29 (60.4%) | 43 (100%) | |
| Delayed graft function* | 19 (39.6%) | 0 (0%) | **<0.001** |
| Up to day 10 | | | |
| Dialysis ($n$) | 19 | 0 | **<0.001** |
| Rejection ($n$)† | 10 | 5 | 0.271 |
| Serum creatinine (mg/dl)‡ | 2.7 (1.6–5.0) | 1.8 (1.2–2.0) | **<0.001** |
| MDRD (ml/min/1.73 $m^2$)‡ | 24.6 (11.6–37.5) | 40.3 (29.7–59.1) | **<0.001** |
| CKD-EPI (ml/min/1.73 $m^2$)‡ | 27.5 (12–44.2) | 42.3 (30.9–64.0) | **<0.001** |
| Up to day 30 | | | |
| Dialysis ($n$) | 19 | 1 | **<0.001** |
| Rejection ($n$)† | 19 | 6 | **0.009** |
| Serum creatinine (mg/dl)§ | 1.7 (1.2–2.1) | 1.4 (1.2–1.8) | 0.082 |
| MDRD (ml/min/1.73 $m^2$)§ | 41.1 (31–55.1) | 46.0 (38.0–60.2) | **0.042** |
| CKD-EPI (ml/min/1.73 $m^2$)§ | 44.0 (32.7–61.9) | 53.3 (42.8–63.5) | 0.084 |
| Up to day 90 | | | |
| Dialysis ($n$) | 21 | 1 | **<0.001** |
| Rejection ($n$)† | 21 | 9 | **0.026** |
| Serum creatinine (mg/dl)¶ | 1.6 (1.1–2.1) | 1.5 (1.2 -1 .8) | 0.412 |
| MDRD (ml/min/1.73 $m^2$)¶ | 41.0 (32.6–60.8) | 46.0 (38.0–60.2) | 0.273 |
| CKD-EPI (ml/min/1.73 $m^2$)¶ | 42.0 (34.2–65.3) | 49.1 (40.6–62.7) | 0.279 |
| Up to day 180 | | | |
| Dialysis ($n$) | 22 | 1 | **<0.001** |
| Rejection ($n$)† | 24 | 14 | 0.09 |
| Serum creatinine (mg/dl)** | 1.6 (1.2–2.5) | 1.5 (1.2–1.8) | 0.367 |
| MDRD (ml/min/1.73 $m^2$)** | 38.9 (25.8–59.9) | 47.0 (39.5–54.8) | 0.247 |
| CKD-EPI (ml/min/1.73 $m^2$)** | 42.0 (26.7–64.5) | 50.7 (41.7–57.8) | 0.229 |

CRP, C-reactive protein; MDRD, modification of diet in renal disease, CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

Data are presented by median and interquartile range (Q1–Q3). A $P$-value (bold values) <0.05 was considered statistically significant.

*Low diuresis (<1000 ml excretion/day) under forced medical stimulation and/or dialysis up to day 7.

†Borderline or acute rejections.

‡At day 10.

§At day 30.

¶At day 90.

**At day 180.



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**Table 2.** Different biomarker levels in patients following kidney transplantation with a graft from a deceased donor (DD) or a living donor (LD) during study participation (Pre – day 10).

| | Pre | | Day 0 | | Day 1 | | Day 3 | | Day 5 | | Day 7 | | Day 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Timepoints | DD | LD | DD | LD | DD | LD | DD | LD | DD | LD | DD | LD | DD | LD |
| Inflammation and acute-phase proteins | | | | | | | | | | | | | | |
| CRP [mg/l] | 2.5 | 2.2 | 4.8 | 2.0 | 46.8 | 25.1 | 27.9 | 19.7 | 13.2 | 8.5 | 8.7 | 10.0 | 6.1 | 1.1 |
| | (2.0–8.5) | (2.0–5.7) | (2.0–11.4) | (2.0–7.3) | (23.7–63.5) | (19.1–40.0) | (21.2–44.5) | (13.0–27.4) | (8.9–18.4) | (5.3–21.2) | (5.7–11.9) | (5.9–13.6) | (3.0–22.9) | (0.2–2.0) |
| | 0.399 | | **0.034** | | **0.001** | | **0.006** | | 0.178 | | 0.929 | | **0.026** | |
| sRAGE | 2104 | 2111 | 1663 | 1694 | 1712 | 1066 | 1792 | 1106 | 1992 | 869 | 1303 | 672 | 1392 | 772 |
| [pg/ml] | (1647–3142) | (1488–3058) | (1302–2508) | (1255–2439) | (1065–2515) | (740–1506) | (989–2807) | (722–1566) | (1051–2887) | (636–1259) | (895–1571) | (523–1269) | (785–1882) | (600–1149) |
| | 0.791 | | 0.768 | | **0.002** | | **0.022** | | **0.001** | | **0.046** | | 0.258 | |
| Cell death biomarkers | | | | | | | | | | | | | | |
| cck18 | 98 | 98 | 88 | 99 | 79 | 77 | 94 | 87 | 109 | 127 | 119 | 129 | 93 | 86 |
| Plasma [U/l] | (75–136) | (75–190) | (75–120) | (75–153) | (75–106) | (75–89) | (75–150) | (75–108) | (81–159) | (91–179) | (75–184) | (80–213) | (77–154) | (75–111) |
| | 0.381 | | 0.280 | | 0.587 | | 0.063 | | 0.474 | | 0.455 | | 0.595 | |
| cck18 | | | 166 | 112 | 124 | 117 | 116 | 147 | 128 | 140 | 179 | 179 | 191 | 137 |
| Urine [U/l] | | | (83–636) | (90–146) | (88–297) | (78–178) | (76–167) | (117–228) | (77–346) | (121–263) | (101–227) | (119–241) | (119–297) | (107–213) |
| | | | 0.166 | | 0.528 | | **0.017** | | 0.532 | | 0.647 | | 0.462 | |

DD, deceased donor transplantation; LD, living donor transplantation; CRP, C-reactive protein; sRAGE, soluble receptor for advanced glycation end products; ccK18, caspase-cleaved keratin 18.

Data are presented by median and interquartile range (Q1–Q3). A P-value (bold values) < 0.05 was considered statistically significant.

optimism-corrected, monoparametric ROC analyses were performed with plasma levels of total K18 as well as urine concentrations of [TIMP-2] × [IGFBP7] of all participating patients at d0 as well as d1. Plasmatic total K18 was shown to be of help for the prediction of a delayed graft function within the first 24 h following kidney transplantation [d0: ROC-area under the curve (AUC) = 0.541, (95% CI = 0.400; 0.682); d1: ROC-AUC = 0.689, (95% CI = 0.571; 0.807)], whereas urinary [TIMP-2] × [IGFBP7] failed to be of prognostic value within the same timeframe [d0: ROC-AUC = 0.431, (95% CI = 0.282; 0.580); d1: ROC-AUC = 0.406, (95% CI = 0.262; 0.550)]. (ii) *Multiparametric ROC analyses*: With regard to the prediction of a DGF, an additional ROC analysis was performed with a routinely used clinical scoring system (including donor age, donor creatinine, recipient body mass index (BMI) and induction therapy), resulting in a ROC-AUC of 0.702. A combined use of this scoring system with biomarkers of cell cycle arrest and/or cell death resulted in an improved identification of patients suffering from a DGF following kidney transplantation (Table 3).

### Graphical abstract

An additional graphical abstract summarizes the results of the presented investigation and is presented in the supplementary data section (Fig. S2).

## Discussion

The present investigation was able to demonstrate that cell death and cell cycle arrest biomarkers are potentially suitable to improve early identification of patients at high risk for the development of a DGF following kidney transplantation from deceased or living donors.

The extent of the IRI is one of the most important influencing factors for early graft function in patients undergoing kidney transplantation: (i) As a result of lacking oxygen supply and adenosine triphosphate depletion, as well as a diminished elimination of toxic metabolites, the ischaemic injury induces necrotic cell death in the graft. (ii) During the subsequent reperfusion procedure, further inflammatory responses are initiated, resulting in an increased cell death due to apoptosis as well as necrosis. Accordingly, the extent of IRI is clearly associated with the incidence of delayed graft functions in patients undergoing kidney transplantation and therefore represents an important measure for the prediction of graft outcome in these patients [13–16]. The extent of IRI should be assessed as early as possible to adapt the

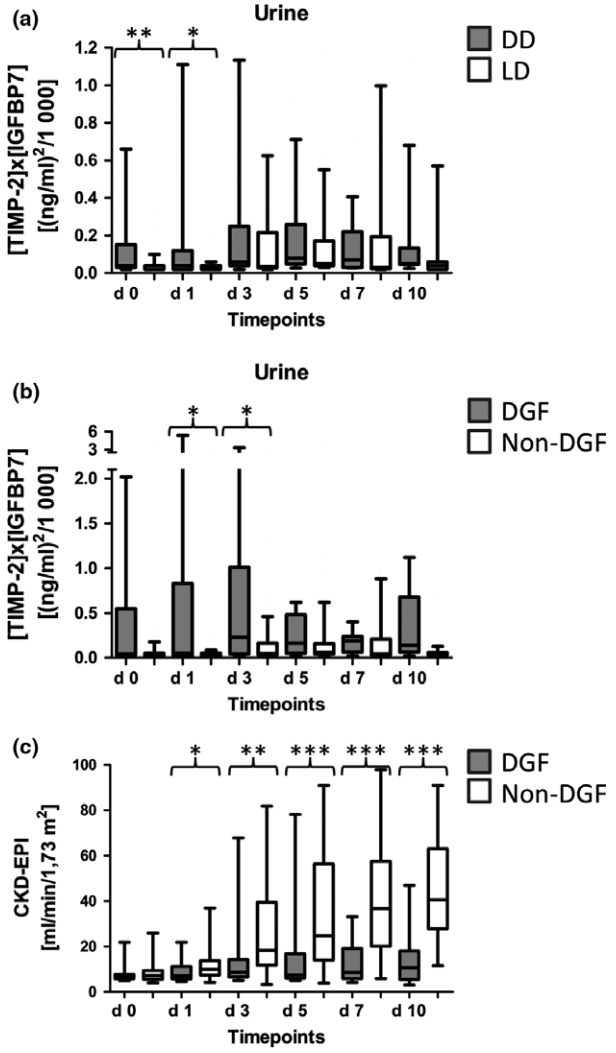


**Figure 1** Urinary concentrations of tissue inhibitor of metalloproteinase-2 (TIMP-2) and insulin-like growth factor-binding protein-7 (IGFBP-7), as well as corresponding glomerular filtration rates in patients following kidney transplantation. Urinary concentrations of [TIMP-2] × [IGFBP7] were measured (a) in patients undergoing deceased donor (DD; grey-shaded bars) or living donor (LD; white bars) kidney transplantation as well as (b) in patients suffering from a delayed graft function (DGF; grey-shaded bars) or with a normal early graft function (Non-DGF; white bars). (c) Moreover, corresponding glomerular filtration rates were estimated by the use of the CKD-EPI (chronic kidney disease epidemiology collaboration)-formula, a creatinine-based formula in patients suffering from a delayed graft function (DGF; grey-shaded bars) or with a normal early graft function (Non-DGF; white bars). Plasma and urine samples were collected prior to transplantation (Pre), immediately after the end of the surgical procedure (d0), and 1 day (d1), 3 days (d3), 5 days (d5), 7 days (d7) and 10 days (d10) afterwards. Data in box plots are given as median, 25th percentile, 75th percentile with the 10th as well as 90th percentile at the end of the whiskers. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

therapeutic concept as soon as possible, including a thorough adjustment of blood pressure and blood sugar levels. Moreover, potentially nephrotoxic substances should be avoided resolutely [17].

To investigate the degree of IRI as well as effects on early graft function, two study groups with different cold ischaemia times were evaluated. The first group underwent deceased donor transplantation (DD: $n = 48$), whereas the second group underwent living donor transplantation (LD: $n = 43$). Concerning donor characteristics of both groups, there were only slight differences concerning donor age and SCr between the two study groups. However, due to a relevant transfer time in DD graft recipients, CIT was shown to be significantly prolonged in these patients. The resulting IRI is known to be a potent inductor of innate immune reactions, which can be demonstrated by the upregulation of pattern recognition receptors (PRR), the release of various cytokines as well as an elevated production of reactive metabolites [e.g. reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive carbonyl species (RCS)] [18,19]. An important member of the PRR family is the receptor for advanced glycation end products (RAGE), which is only expressed to a limited extent under physiological conditions, but can be induced by binding of its ligands (e.g. high mobility group box protein-1, amyloid ß, AGEs and S100 proteins). Ligation of RAGE may lead to the perpetuation of nuclear factor kappa-B activation, resulting in a propagation of immunoinflammatory responses [20–22]. The relevance of RAGE-mediated inflammation in the context of IRI has already been described in patients undergoing orthotopic liver transplantation [18]. However, these results also seem to hold true for patients following kidney transplantation. Plasma levels of sRAGE were significantly elevated up to 7 days after the transplantation procedure in patients of the DD group in comparison with LD graft recipients. Within the context of innate immunity, acute-phase reactions (as assessed by CRP plasma levels) were also shown to be significantly increased in patients undergoing deceased donor transplantation, giving further evidence for a higher degree of the immunoinflammatory response in these patients.

C-reactive protein and sRAGE results clearly indicate that the duration of CIT is closely related to the degree of the inflammatory response, and the resulting extent of IRI might be able to affect graft integrity in a "dose-dependent" manner. Accordingly, DD graft recipients suffered more frequently from a delayed graft function in combination with higher serum creatinine levels and

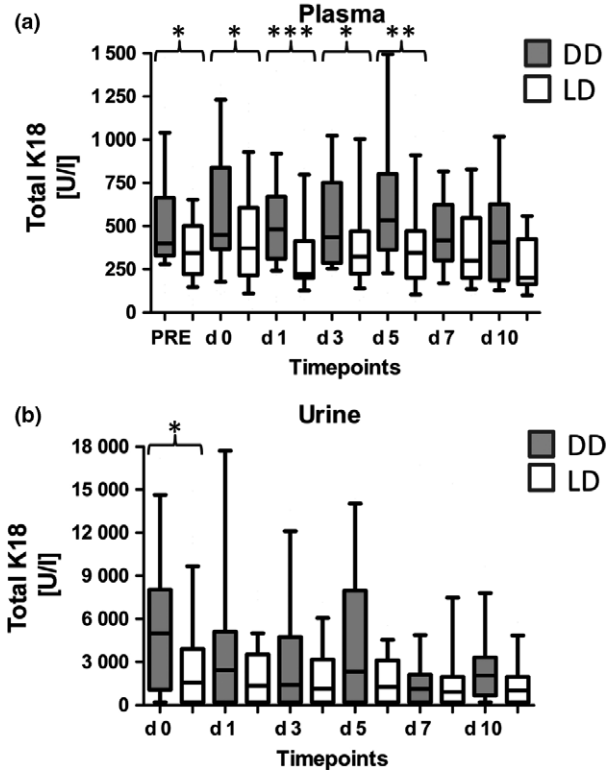


**Figure 2** Total keratin 18 (total K18) levels in (a) plasma and (b) urine of patients following kidney transplantation with a graft from a deceased donor (DD; grey-shaded bars) or a living donor (LD; white bars). Plasma and urine samples were collected prior to transplantation (Pre), immediately after the end of the surgical procedure (d0), and 1 day (d1), 3 days (d3), 5 days (d5), 7 days (d7) and 10 days (d10) afterwards. Data in box plots are given as median, 25th percentile, 75th percentile with the 10th as well as 90th percentile at the end of the whiskers. *p < 0.05; **p < 0.01; ***p < 0.001.

**Table 3.** Multiparametric ROC analyses.

| | | ROC-AUC |
|---|---|---|
| Clinical scoring system* | | 0.702 |
| Timepoint | d0 | d1 |
| Clinical scoring system* + CKD-EPI | 0.704 | 0.774 |
| Clinical scoring system* + [TIMP-2] × [IGFBP7] | 0.645 | 0.741 |
| Clinical scoring system* + total K18 plasma | 0.813 | 0.863 |
| Clinical scoring system* + [TIMP-2] × [IGFBP7] + total K18 plasma | 0.744 | 0.861 |

AUC, area under the curve; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; K18, keratin-18; ROC, receiver operating characteristic; [TIMP-2] × [IGFBP7], tissue inhibitor of metalloproteinase-2 and insulin-like growth factor-binding protein-7.

*Clinical scoring system: donor age, donor creatinine, recipient body mass index (BMI) and induction therapy.

lower glomerular filtration rates. In contrast, LD graft recipients showed an unobtrusive immediate graft function from d0 up to d30 in all cases. However, these IRI-associated functional impairments in DD graft recipients appeared to be a transient effect, especially in the early phase following kidney transplantation. In the following two investigation periods (up to day 90 and day 180), the two study groups did not differ significantly with regard to graft integrity as well as the need for RRT, indicating an at least partial recovery of the graft. Nevertheless, to provide best supportive graft care and to avoid additive harmful effects (e.g. nephrotoxic drugs, poor hemodynamic circumstances, inadequate fluid balance), early graft dysfunctions need to be detected as early as possible. Within this context, biomarkers of cell cycle arrest, as well as cell death, might be of important value.

TIMP-2 and IGFBP7 both represent biomarkers for cellular stress and are expressed in the early phase after renal tubular damage. They are both involved in the G1 cell cycle arrest, disabling the damaged cells to perform cell division. Urinary concentrations of [TIMP-2] × [IGFBP7] have proven to be early and reliable markers for an acute kidney injury (AKI) in mixed intensive care populations [23–25] as well as in different postoperative settings (e.g. major surgery, cardiac surgery) [26,27]. Moreover, these biomarkers have recently been shown to be of value for the prediction of a delayed graft function in patients following deceased donor transplantation [11]. In line with the investigation of Pianta *et al.*, urine levels of [TIMP-2] × [IGFBP7] were shown to be significantly increased in patients suffering from a DGF within the presented investigation. Moreover, [TIMP-2] × [IGFBP7] measurements were demonstrated to be at least comparable (e.g. CKD-EPI) or superior (e.g. MDRD) to the most widely used creatinine-based estimates for the glomerular filtration rate. This is due to the fact that urine levels of [TIMP-2] × [IGFBP7] reached the best discriminative value already at very early stages following kidney transplantation (at d0 or d1), whereas a creatinine-based estimation of the glomerular filtration rate showed significant differences not until d1. However, although urine levels of [TIMP-2] × [IGFBP7] differed significantly between patients with or without DGF at early stages following kidney transplantation, an additional diagnostic value of [TIMP-2] × [IGFBP7]

measurements could only be observed in combination with a routinely used clinical scoring system [6].

Patients with a severe chronic kidney disease are characterized by an increased cell death, and keratin measurements in plasma and urine have proven to be useful within this context [15,28,29]. Keratins belong to the family of intermediate filament proteins and are common in both epithelial and endothelial cells [30]. Depending on the type of cell death (apoptosis versus necrosis), specific K18 isoforms (caspase-cleaved vs. full-length, uncleaved K18) are released, which can then be detected by specific antibodies: (i) During apoptosis, the organism attempts to use a highly regulated system to make cell death as less harmful as possible for the organism [31]. During the decomposition processes, various ATP-dependent caspases are activated leading to the cleavage of K18 into characteristic fragments (ccK18), which can then be detected by the M30 antibody. Unfortunately, data concerning the role of caspases are conflicting. On the one hand, caspases are involved in the process of cell proliferation and differentiation [32,33]. On the contrary, the extent of apoptosis seems to be closely related to the disease severity in some chronic liver diseases [34,35]. (ii) In contrast, several disease states (e.g. ischaemia and severe tissue trauma) might result in rapid and undirected necrotic cell death [36], which is anything but harmless due to its severe proinflammatory side effects. Necrosis is known to be caspase-independent leading to a release of full-length K18 into plasma/urine. The M65 Epideath-ELISA is able to detect all K18 isoforms, indicating the extent of total cell death. A combined use of both assays (M30, M65) is able to directly assess the proportion of apoptosis in relation to total cell death, whereas the extent of necrosis can be estimated indirectly. Within the presented investigation, an elevated inflammatory response in patients of the DD group was paralleled by significantly increased levels of total K18 in plasma up to d5. Due to comparable plasmatic levels of ccK18 in the DD and LD graft recipients, necrosis seems to be the predominant mode of cell death in patients of the DD group resulting in the above-described increased levels of total K18. Moreover, the extent of necrotic cell death was shown to be directly associated with graft integrity, as total K18 monitoring was identified to be a suitable tool for the detection of delayed graft function in patients following kidney transplantation. The diagnostic value of total K18 monitoring was further increased by its combined use with a routinely used clinical scoring system [6]. Contrariwise, cell death caused by apoptosis appears to have a merely subordinate role in these patients, so that ccK18 monitoring was shown to be of minor value. Also, plasma levels of total K18 in the DD group were shown to be significantly elevated even before the operation (at Pre), potentially indicating a longer lasting and more severe chronic kidney disease before kidney transplantation. This hypothesis is further supported by the fact that patients of the DD group were significantly older and required RRT in a higher number of cases. This is in line with the results from Roth *et al.*, who were able to show that patients with advanced chronic renal failure are hallmarked by elevated total K18 values [29].

### Limitations

There are several limitations, which need to be addressed in connection with the presented manuscript: First, this is a single-centre study in a relatively small patient cohort. Therefore, the study design appeared to be unsuitable for a reliable calculation of cut-off values of the reported biomarkers for early prediction of DGF in patients following kidney transplantation. Second, at the start and over the course of the study neither imaging procedures to calculate the nephron mass nor additional biopsy samples to assess the organ quality were taken routinely unless we suspected organ rejection. Thus, a varying organ quality cannot be ruled out completely as a contributing factor for DGF development and divergent graft outcomes, besides CIT. Furthermore, we cannot make any statements regarding the long-term survival of the grafts. The study was designed for only a period of 180 days.

## Conclusion

To the best of our knowledge, this is the first clinical study directly assessing the effects of prolonged CIT and increased IRI on graft dysfunction due to cell cycle arrest and necrotic cell death. As a result of prolonged CIT and increased IRI, deceased donor transplantations were shown to suffer from a more distinct cell cycle arrest and necrotic cell death. Plasmatic total K18 and urinary [TIMP-2] × [IGFBP7] were therefore demonstrated to be of value for the detection of a DGF, as they improved the diagnostic performance of a routinely used clinical scoring system (including donor age, donor creatinine, recipient body mass index (BMI) and induction therapy). However, prior to their implementation into clinical routine, further adequately powered investigations with high-quality data collection need to be recommended.

## Authorship

FCFS: conceived of the study, participated in its design and coordination, and helped to draft the article. Furthermore, he performed data acquisition, carried out the enzyme-linked immunosorbent assay measurements in the laboratory and prepared the tables and figures. ES and JF: performed data acquisition and were involved in revising the article critically. TF, TS, JZ, FU, LK, CN, CM and MZ: participated in the design of the study and have been involved in revising the article critically. TB: participated in the design of the study and performed statistical analysis. Furthermore, he was involved in revising the article critically. AM, PPN and MAW: participated in the design of the study and have been involved in revising the article critically. SH and TB: conceived of the study, participated in its design, coordinated and helped to draft the article.

## Funding

This study was carried out with financial resources of the Department of Anesthesiology (Heidelberg University Hospital, Heidelberg, Germany), the Department of General, Visceral and Transplant Surgery (Heidelberg University Hospital, Heidelberg, Germany), Department of Internal Medicine I and Clinical Chemistry (University Hospital Heidelberg, Heidelberg, Germany), the Department of Nephrology (Heidelberg University Hospital, Heidelberg, Germany) and the Institute of Medical Biometry and Informatics (University of Heidelberg, Heidelberg, Germany). Moreover, this investigation received a financial grant from the Heidelberg Foundation of Surgery.

## Conflict of interests

The authors declare no conflict of interests.

## Acknowledgements

The authors gratefully acknowledge U. Krauser for her excellent technical assistance. Furthermore, Thomas Fleming, Johanna Zemva and Peter P. Nawroth were supported by SFB 1118.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Glomerular filtration rates were estimated by the use of the MDRD (Modification of Diet in Renal Diseasea)-formula, a creatinine-based formula in patients suffering from a delayed graft function (DGF; grey-shaded bars) or with a normal early graft function (Non-DGF; white bars).

**Figure S2.** The graphical abstract gives a brief overview about the main results of this study.

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