Live Confocal Tissue Assessment With SYTO16/PI and WGA Staining Visualizes Acute Organ Damage and Predicts Delayed Graft Function in Kidney Transplantation

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Objective: The aim of our prospective clinical trial was to test a tissue staining technique (real-time confocal analysis [RTCA]) as a rapid assessment tool for donor kidney quality and function in human kidney transplantation.

Summary Background Data: Tools for objective graft tissue viability assessment before kidney transplantation are lacking. RTCA has recently been established and tested in a pilot study using rodent kidneys.

Methods: RTCA was performed in kidney biopsies stained with SYTO16/PI and WGA. A score between −3 (100% nonviable) and +3 (100% viable) describes the sum of viable cells divided by the number of nonviable cells per examined area (glomerulus, proximal, and distal tubules). The primary study endpoint was the delayed graft function (DGF).

Results: Seventy-one kidney transplant recipients were transplanted. The median recipient and donor age were 58.5 and 57 years, respectively. Cold ischemia time was 13.6 ± 4.7 hours; anastomosis time was 30.8 ± 8.7 minutes (mean ± SD). Overall, 23 (33.8%) patients developed DGF. The RTCA score was significantly lower in kidneys developing DGF −0.43 ± 1.78 versus no DGF 0.91 ± 2.17, $P = 0.01$. The Remuzzi score did not differ between DGF and no DGF, $P = 0.13$. Remuzzi score and RTCA score correlate inversely significantly; $P = 0.004$. In the multivariate analysis, solely RTCA score was revealed as a significant independent factor predicting DGF; $P = 0.015$, Wald = 5.95, odds ratio = 0.72, 95% confidence interval = 0.55 to 0.94.

Conclusions: Our data demonstrate that RTCA is feasible and clinically meaningful. The RTCA score predicts DGF and is a valid option to be applied in renal transplantation.

Keywords: assessment, confocal microscopy, delayed graft function, kidney transplantation, outcome

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Delayed graft function (DGF), often referred to as acute kidney injury (AKI) of the transplanted kidney,[1] occurs in up to >50% of all transplanted kidneys depending on the proportional distribution of living and deceased donor kidneys in a transplant program.[1–5] In the clinical routine, DGF is most commonly defined as the need for dialysis within the first week after transplantation.[6,7] The major impact of DGF related to the immediate clinical condition includes an increased morbidity and a prolonged hospital stay leading to increased costs.[2,8,9] Furthermore, DGF is one of the most important risk factors for biopsy-proven acute rejection (BPAR).[10] This is particularly problematic because rejection may remain undetected in patients suffering from DGF.[11]

In addition to the immediate effects during the early period after transplantation, DGF is an independent risk factor for inferior graft survival long term.[2,3,8,12–16] The majority of tools predicting DGF are based on donor and graft characteristics. The conventional clinical and histological tools, however, fall short in actually assessing the quality and viability of the graft parenchyma and specific compartments of the kidney[5,17–20] Histopathological analysis of pretransplant biopsies is time consuming and remains insufficient with the intention to predict DGF.

With the ongoing trend to accept more extended criteria donor (ECD) organs for transplantation, the need for a more accurate assessment of organ quality is a pressing issue. The condition of the donor organ, the condition of the recipient, and the injury occurring during ischemia and preservation define the eventual outcome. The projections made by clinicians are based on the sum and individual weight of parameters known to be associated with the outcome.

The aim of our prospective clinical trial was to establish a rapid assessment tool of donor kidney quality, using live tissue staining with SYTO16, propidium iodide (PI), and wheat germ agglutinin (WGA), and investigate its value to predict DGF in human kidney transplantation using real-time confocal analysis (RTCA).

METHODS

Clinical Trial Design

Based on a previously established technology (real-time confocal analysis of SYTO16/PI and WGA) using rodent kidneys,[21,22] a prospective observational single arm clinical trial was conducted at the Medical University of Innsbruck between October 2015 and July 2017. The study was approved by the institutional review board of the Medical University of Innsbruck (AN2015-0101 348/4.25) and all patients signed an informed consent form.

Seventy-one consecutive patients were included in this trial. The biopsy taken for tissue sampling is part of an established routine. As RTCA does not require slicing of the tissue sample, no additional biopsy was required. After RTCA, samples were used for routine histologic assessment. Recipient, donor, and transplant characteristics/

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data pretransplant and during the postoperative course were collected and collated. Delayed graft function was defined as the need for at least one dialysis within the first 7 days after transplantation with the exception of dialysis for hyperkalemia or hypervolemia within the first 12 hours posttransplant.

Sampling and Preparing Kidney Biopsies for Real-time Live Confocal Imaging

Kidney biopsies were taken immediately after unpacking and starting the back-table preparation of the organ. A punch biopsy (4 mm diameter) was used to obtain standardized cylindrical tissue samples of approximately 8 to 10 mm length. The samples were then immediately placed in Custodiol HTK Solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany).

Real-time live confocal microscopy assessment was performed using the following live stains: WGA conjugate (Molecular Probes, Eugene, OR; 10 μg/mL final concentration) stains the plasma membrane showing tissue morphology, SYTO16 (Molecular Probes; final concentration 5 μM) binds to all nuclei of vital cells and PI (Molecular Probes; final concentration 500 nM) binds nuclei of dead cells.[23]

Real-time live confocal imaging was performed in 8 well-chambered cover glasses (Nalge Nunc International). Confocal images were acquired with a spinning disk confocal system (UltraVIEW VoX; Perkin Elmer, Waltham, MA) connected to a Zeiss Axio Observer Z1 microscope (Zeiss, Oberkochen, Germany). Images were acquired with the Volocity software (Perkin Elmer), using a 10× objective. Images shown are z-stacks of 10 planes with a spacing of 4 μm. Incubation time was 15 minutes at 37°C; the readout was performed in 5 minutes.

Real-time Confocal Microscopy Readout

In each kidney biopsy, 10 optical sections of 1 μm were analyzed. Live and dead cells in the kidney biopsy were quantified using the dyes SYTO16/PI and WGA.

Cell viability and matrix architecture in the kidney cortex were quantified by counting events (one event is either a live or a dead cell) and groups which comprise (1) cells from the glomerulus; (2) cells from the proximal tubules; and (3) cells from the distal tubules.

For each section, the number (total count) of viable cells was divided by the number of dead cells (total count) with following possible results: (+1) for highly viable biopsies/areas with more viable than dead cells; (0) for biopsies/areas in which the number of viable cells equals the one of dead cells; (−1) for those in which the number of dead cells outnumbers the one of live cells. For each biopsy, a score was calculated consisting of the glomerular and the 2 tubular areas resulting in a maximum of +3 points in the best or −3 in the worst-case scenario.

Sampling and Handling of Kidney Biopsies for/and Conventional Histology

After completion of live confocal imaging, the kidney biopsy was placed and fixed in Millonig's solution and processed for paraffin embedding. Three-micrometer-thick sections were stained by hematoxylin and eosin. Light microscopy observations were carried out on a Nikon Eclipse 50i microscope (Nikon Corporation, Japan). Histological assessment was performed according to the Remuzzi scores.[24]

Statistical Analysis

The statistical testing for the pilot trial was done with Graph Pad Prism 7 and IBM SPSS Statistics Version 25. A P value of <0.05 was considered as statistically significant. Biopsy results (RTCA and Remuzzi scores), recipient, donor, and transplant factors were analyzed using parametric and nonparametric tests (including Spearman rank correlation). The RTCA score adjusted for different clinically relevant parameters was further evaluated in multivariate logistic regression analyses. ROC analyses for RTCA and Remuzzi scores were performed (Figs. 1 and 2).

RESULTS

Seventy-one kidney transplant patients have been recruited and successfully transplanted; of those, 19 (26.8%) were female and 14 (19.7%) patients received a retransplantation. Three biopsies could not be included in the real-time analysis due to technical and/or logistical failures. Data from these 3 cases were not included in the statistical analysis. Table 1 depicts the demographics and the transplant factors of 68 kidney transplants performed. All biopsies taken met the Banff adequacy criteria.[25]

RTCA scoring required approximately 30 minutes for completion including readout, hence the method proved to be very feasible for immediate assessment.

Delayed Graft Function, RTCA, and Remuzzi Scores

Twenty-three recipients (23/68, 33.8%) suffered from delayed graft function after kidney transplantation. The DGF rate was proportionally but not statistically significantly higher in retransplantations (7/14, 50%) compared with first transplants (16/54, 29.6%), $P = 0.21$. The proportion of ECD kidneys was numerically, but not significantly, higher in patients developing DGF posttransplant [60.9% (14/23) in DGF vs 42.2% (19/45) no DGF, $P = 0.2$].

The Remuzzi scores were insignificantly lower in biopsies of organs without DGF; 1.36 ± 1.56 no DGF versus 2.14 ± 1.96 in DGF, $P = 0.13$. In contrast, the RTCA score was significantly higher in kidneys with initial graft function; no DGF 0.91 ± 2.17 confocal score versus -0.43 ± 1.78 in DGF, $P = 0.01$.

Comparison of confocal and Remuzzi scores of one and the same biopsy area revealed a significant and inverse correlation of the 2 scores: the higher the confocal score, the lower the Remuzzi score and vice versa; $P = 0.004$, Spearman's rho correlation coefficient was −0.32. This finding is indicating that RTCA offers a clinical meaningful assessment.

Risk Factors for DGF

In line with the results displayed in Table 2, univariate logistic regression analyses to detect predictors for DGF yielded only the RTCA score ($P = 0.01$) as statistically significant. Table 2 illustrates the differences between kidneys with initial (n = 45) and delayed (n = 23) graft function.

Table 3 shows the results of 3 different models (A, B, and C) that adjusted the RTCA score multivariately for relevant demographic and transplant-specific parameters. In consideration of the overall relatively small cohort and the limitations of the regression models, we were not able to present a model that included all parameters reported as important factors in the literature simultaneously. However, in all variations of the number of variables taken into the calculation for the multivariate analyses, the RTCA score remained the sole independent, predictive factor for DGF.

To determine the value of both scores to predict DGF, ROC analyses were performed and revealed the following: The ROC for the Remuzzi score shows a c-index of 0.61 [95% confidence interval (CI), 0.46–0.76], whereas the ROC for RTCA reveals significance with a c-index of 0.69 (95% CI, 0.56–0.81). This further underlines the predictive value of RTCA toward DGF.

Correlation With 3- and 12-month eGFR

Logistic regression analyses detected KDPI [$P = 0.026$, odds ratio (OR) 0.968, 95% CI, 0.94–0.99, Wald 4.954] and sex of the recipient ($P = 0.018$, OR 5.059, 95% CI, 1.33–19.29, Wald 5.638) as

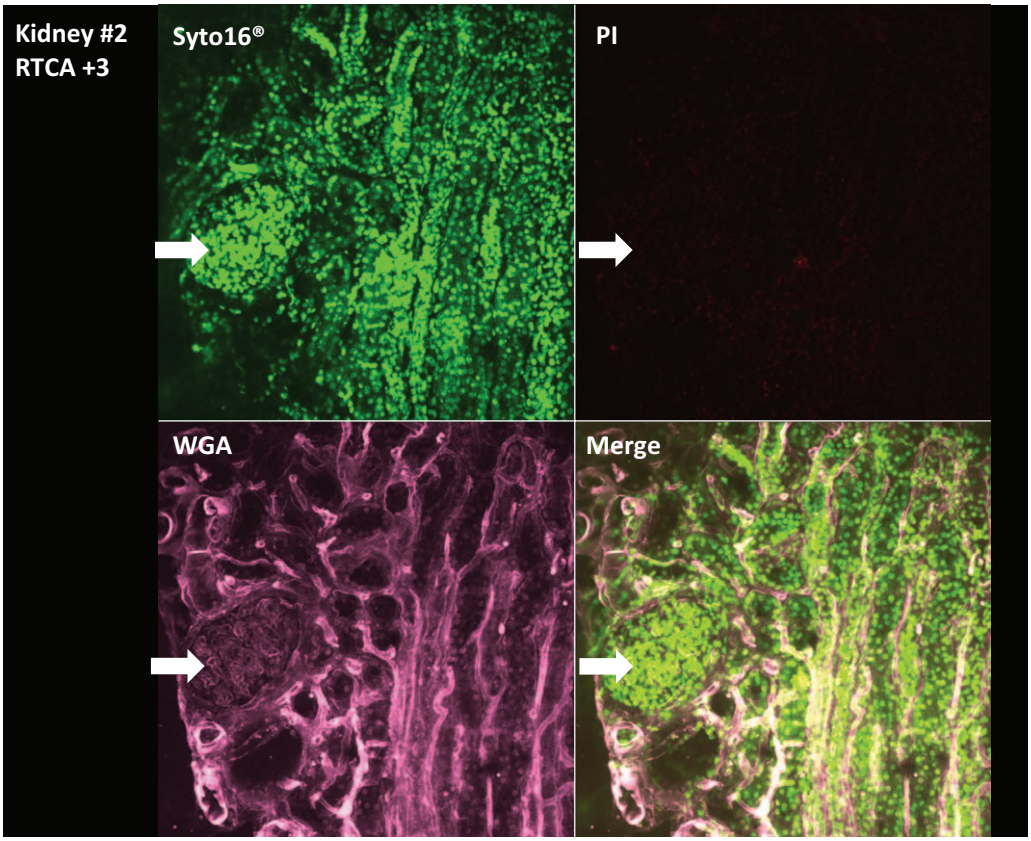

FIGURE 1. Live confocal microscopy photographs of kidney #2: 62-year old, female, extended criteria donor kidney with 20 hours 35 minutes cold ischemia time. Remuzzi score was 2. Kidney developed initial graft function. The arrow indicates the glomerulum.

important significant factors for a 3-month eGFR $\geq$60 mL/min/1.73m^2; female kidney transplant recipients had a higher eGFR 3 months posttransplant.

Donor age ($P = 0.011$, OR 0.907, 95% CI, 0.84–0.98, Wald 6.48) and HLA-DR mismatches ($P = 0.013$, OR 12.25, 95% CI, 1.68–89.43, Wald 6.107) were independent and significant factors influencing 12-month eGFR.

Calculations along the logistic regression model for 3- and 12-month eGFR did not indicate any of the biopsy scores (RTCA and Remuzzi) as significant, for neither of the 2 time points.

Relation Between RTCA Score and Urine Albumin to Creatinine Ratio

One of the most important predictors for graft survival and long-term kidney function after kidney transplantation is proteinuria. Proteinuria is mainly caused by glomerular disease in native kidneys but predominantly of tubular origin posttransplant.[8,26–28] To further investigate the predictive value of the RTCA score, the difference between the 3- and 12-month urinary albumin to creatinine ratio (ACR) (in mg/g) was analyzed and compared within kidney groups with a confocal score between "0" and "−3" and kidneys with a confocal score between "1" and "3". The differences between 3- and 12-month ACR were correlated with DGF and Remuzzi scores between "0" and "2" and equal/more than "3".

There was no difference in regard to an increase or decrease of ACR in kidneys with or without DGF between months 3 and 12 posttransplant (mean ACR -22.4 ± 44.1 mg/g without DGF vs 15.4 ± 590.6 mg/g with DGF, $P = 0.22$).

Kidneys with a Remuzzi score $\geq$3 did not show a different course with respect to ACR dynamics between months 3 and 12 after transplant compared with kidneys with a Remuzzi score $\leq$2 (high Remuzzi score mean ACR -163.6 ± 521.5 mg/g vs mean ACR 71.08 ± 302.2 mg/g in low Remuzzi scores, $P = 0.38$).

In contrast, kidneys with a confocal score $\geq$1 correlate significantly with an ACR decrease between months 3 and 12 posttransplant when compared with kidneys diagnosed with a confocal score $\leq$0 (high RTCA score mean ACR -130.8 ± 436.8 mg/g vs mean ACR 101.9 ± 330.9 mg/g in kidneys with a low RTCA score, $P = 0.039$).

DISCUSSION

In this prospective pilot study, we present the first clinical application of a newly developed RTCA score in kidneys accepted for transplantation offering the excellent possibility to judge parenchyma viability and predict DGF.

Over the last 2 decades, a number of scoring systems were developed aiming to estimate the quality of a donor kidney and to predict organ function in the recipient after transplantation.[5,19,29,30] Scores like KDRI and KDPI are useful tools to estimate the relative risk for posttransplant kidney graft failure.[29,31] Such scores, however, have to be used with caution. Bae et al revealed that evidently some viable kidneys were inappropriately discarded after the implementation of the KDPI/KDRI scores in the United States.[32] As recipients of SCD/high-KDPI kidneys have a decreased mortality in comparison to those who remained on the waiting list, the KDPI has to be used

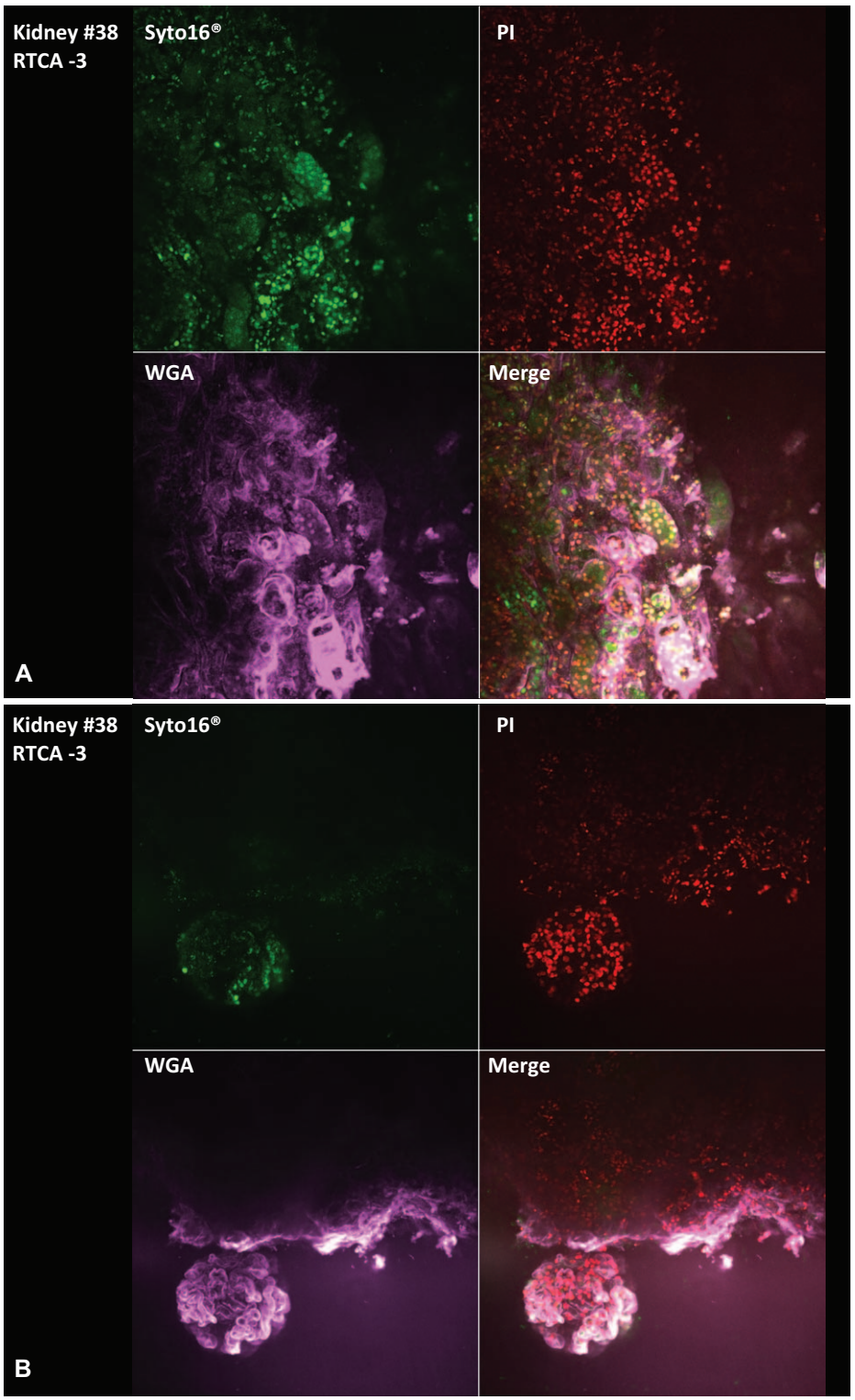

FIGURE 2. (A) Live confocal microscopy photographs of kidney #38; tubular structures: 56-year-old, male, extended criteria donor kidney with 13 hours 28 minutes cold ischemia time. Remuzzi score was 4. Kidney developed delayed graft function. (B) Live confocal microscopy photographs of kidney #38; glomerulum: 56-year-old, male, extended criteria donor kidney with 13 hours 28 minutes cold ischemia time. Remuzzi score was 4. Kidney developed delayed graft function. For Figures 1 and 2A, B, we used the following stains: (1) Syto16, which stains nuclei in dead and living cells, (2) propidium iodide (PI), which is only taken up by nuclei of dead cells, and Alexa647 coupled wheat germ agglutinin conjugate (WGA), which binds to *N*-acetyl-D-glucosamine and *N*-acetyl-D-neuraminic acid residues, thereby staining cell membranes as well as matrix components and thus can be used to monitor the intactness of the tissue. The merged image shows the precise location of stained cells within the tissue.

TABLE 1. Demographics and Transplant Factors of Kidney Transplants Recipients With Analyzed Biopsies

Characteristics	Overall Cohort (n = 68)*
Recipient age, yrs (median (min–max))	58.5 (29–75.8)
Recipient BMI, kg/m^2 (mean, SD)	27 ± 4.8
Recipient male sex (n, %)	49 (72%)
Prior Tx (n, %)	14 (20.6%)
Donor age, yrs (median (min–max))	56.6 (0.2–81.5)
Donor BMI, kg/m^2 (mean, SD)	26.2 ± 2.2
Donor male sex (n, %)	17 (53%)
Extended criteria donor (ECD) (n, %)	33 (48.5%)
Kidney donor risk index (KDRI) (mean, SD)	1.33 ± 0.43
Kidney donor profile index (KDPI), % (mean, SD)	68.5 ± 23.6
Cause of end-stage renal disease (n, %)	
Glomerulonephritis	20 (29.4%)
Diabetic nephropathy	13 (19.1%)
Hereditary renal disease	11 (16.2%)
Vascular nephropathy	8 (11.8%)
Others	16 (23.5%)
Cold ischemia time, h (mean, SD)	13.6 ± 4.7
Anastomosis time, min (mean, SD)	30.8 ± 8.7
Class I, mm	
HLA A, mm (mean, SD)	1.13 ± 0.64
−0 and 1 (n, %)	49 (72.1%)
−2 (n, %)	19 (27.9%)
HLA B, mm (mean, SD)	1.16 ± 0.7
−0 and 1 (n, %)	45 (66.2%)
−2 (n, %)	23 (33.8%)
Class II, mm	
HLA DR, mm (mean, SD)	1.01 ± 0.61
−0 and 1 (n, %)	55 (88.9%)
−2 (n, %)	13 (19.1%)
Total number, mm (median (min–max))	3 (0–6)

*The overall cohort in the entire results section refers to the cohort of analysed biopsies (n = 68); 71 patients were recruited, but 3 kidney biopsies could not be analyzed due to technical/logistical issues.

considering the fact that the nontransplanted patient will experience all side effects of dialysis otherwise.[32] In fact, the KDRI has statistically a low predictive accuracy and concordance between predicted and actual outcomes for kidneys.[33] In our trial, the calculated KDRI/KDPI did not have any predictive value for the occurrence of DGF or the estimated 12-month GFR.

The alternative approach for organ quality assessment and outcome prediction is based on a morphological assessment of the graft tissue. The evaluation of a kidney zero biopsy to investigate the parenchyma quality pretransplant is the logic choice, but the established tests are limited in their predictive value. The clinically most frequently used scoring system was developed by Remuzzi et al.[24,34] Kidneys with a score of 0 to 3, potentially 4, are considered for transplantation as a single kidney, whereas for organs with a score of 5 to 6 a dual transplant is recommended. Kidneys with a score of ≥7 are suggested to be discarded.[24,33,34] This score is often applied in

TABLE 3. RTCA Score as a Predictor for DGF Adjusted for Clinically Relevant Characteristics in Multivariate Logistic Regression Analyses

***Model A*: Recipient and donor age adjusted results of the multivariate logistic regression model to evaluate predictors for DGF**

Characteristic	Wald	Odds Ratio	95% CI	*P*
Recipient age	0.418	1.017	0.966–1.072	0.518
Donor age	0.124	0.993	0.958–1.030	0.724
RTCA score	5.949	0.721	0.555–0.938	**0.015**

***Model B*: Transplant factor adjusted results of the multivariate logistic regression model to evaluate predictors for DGF**

Characteristic	Wald	Odds Ratio	95% CI	*P*
Prior transplant	0.968	1.906	0.527–6.889	0.325
HLA DR, mm	1.721	1.895	0.729–4.923	0.190
Cold ischemia time	0.153	1.023	0.913–1.145	0.696
Anastomosis time	0.102	1.010	0.948–1.076	0.750
RTCA score	4.823	0.736	0.560–0.968	**0.028**

***Model C*: Remuzzi score and donor risk index adjusted results of the multivariate logistic regression model to evaluate predictors for DGF**

Characteristic	Wald	Odds Ratio	95% CI	*P*
Remuzzi score	0.046	1.042	0.715–1.519	0.830
KDRI/KDPI	1.788	2.733	0.626–11.926	0.181
RTCA score	4.403	0.725	0.537–0.979	**0.036**

RTCA indicates real-time confocal analysis.
Bold values indicate significant values.

TABLE 2. Demographic and Outcome Factors in Kidneys (n = 68) With and Without DGF

Characteristic	DGF (n = 23)	No DGF (n = 45)	*P*
Recipient age, yrs (mean, SD)	57.8 ± 7.7	57.4 ± 12.7	0.900
Recipient BMI, kg/m^2 (mean, SD)	28.5 ± 4.8	26.6 ± 4.7	0.080
Prior Tx (n, %)	7 (30.4%)	7 (15.6%)	0.210
Donor age, yrs (mean, SD)	56.1 ± 19.1	57.3 ± 12.9	0.750
Extended criteria donor (ECD) (n, %)	14 (60.9%)	19 (42.2%)	0.200
Kidney donor risk index (KDRI) (mean, SD)	1.44 ± 0.47	1.28 ± 0.4	0.140
Kidney donor profile index (KDPI), % (mean, SD)	73 ± 27.5	66.2 ± 21.3	0.150
Cold ischemia time, h (mean, SD)	13.9 ± 4.3	13.4 ± 5	0.670
Anastomosis time, min (mean, SD)	32 ± 10	30.2 ± 8	0.610
RTCA score (mean, SD)	−0.43 ± 1.78	0.91 ± 2.17	**0.010**
Remuzzi (mean, SD)	2.14 ± 1.96	1.36 ± 1.55	0.130
Outcome			
eGFR at discharge, mL/min/1.73 m^2 (mean, SD)	32.5 ± 13.9	44.6 ± 11.9	**<0.001**
eGFR 3 mo, mL/min/1.73 m^2 (mean, SD)	40.1 ± 16.8	49.8 ± 14.2	**0.011**
eGFR 12 mo, mL/min/1.73 m^2 (mean, SD)	42.8 ± 16.2	50 ± 14.9	0.160
Graft loss 12 mo (n, %)	1 (4.3%)	0	NA
Patient death 12 mo (n, %)	1 (4.3%)	0	NA

NA indicates not applicable; RTCA, real-time confocal analysis.

the clinical routine despite the several pitfalls and its limited predictive value in single kidney transplantation. The parameters amounting to the Remuzzi score describe chronic changes of the kidney; it does not reflect and account for the additional acute kidney injury occurring in the donor during retrieval and preservation. This is probably also the reason why the score does not show a correlation with the DGF or 12-month eGFR in our analysis. Despite its clear advantages, several limitations of this score have been identified, but alternatives remain to be established.[33,35,36]

Gill et al demonstrated that the risk of death immediately after transplant was highest in recipients who developed DGF when compared with patients remaining on the waiting list and patients after transplantation without DGF.[37] The long-term risk of death was lower with transplantation, but the survival benefit for patient with DGF was achieved at a later point in time.[37] In an earlier assessment in DBD kidneys (n = 1245), we found DGF to be one of the most important independent predictors for graft and patient survival.[38] This vindicates that strategies for early detection and prevention of DGF are needed to reduce graft loss and death after transplantation.[37,38] The application of RTCA for evaluation of kidneys before transplantation revealed a predictive value of this technology for prognosticating initial kidney function after transplantation. The score seems representative for the actual state of the kidney and provides additional information for the responsible physician to adopt clinical care in accordance with the projected DGF risk. This may include adjusting levels of immunosuppression, planning protocol biopsies to timely diagnose acute rejection episodes, fluid management, and other measures. The predictive value of RTCA for DGF is most probably the detection of acute glomerular and tubular damage in addition to preexisting conditions in the organ. With the quality to predict DGF, the RTCA score could become a valuable additional diagnostic asset. Together with the rapid availability of this assessment, RTCA is thought to be a considerable addition to conventional histology. The inverse correlation of RTCA and the Remuzzi score indicates most likely the chronic preexisting damage that is displayed both in conventional histology and RTCA. As RTCA and conventional histology can be performed using the same sample, images of exactly the same areas of the biopsy can be compared. The stains used for confocal analysis are neither toxic, nor influencing the subsequent routine histological and immunohistochemical assessment. The time used for real-time live confocal analysis is <30 minutes and none of the biopsies were damaged or lost due to the staining process.

A limitation of our study is the sample size. The feasibility of evaluating RTCA in a multicentre trial is very reasonable because the technique is relatively easy and the readout reproducible. Another limitation of this trial is that no serial assessment during cold storage has been performed; hence, the impact on preservation on the RTCA score cannot be appraised. Cold ischemia time as a single factor itself did not impact the RTCA score.

The RTCA score may have great value in the context of emerging preservation technologies. With machine preservation eventually allowing for longer term organ preservation and possibly conditioning, more sophisticated tissue viability investigation may become a cornerstone in the pretransplant organ assessment.

To summarize, using SYTO16/PI and WGA to stain kidneys before transplant was a feasible and safe approach. The real-time confocal analysis score was found to be an independent significant predictor for the occurrence of DGF after DBD kidney transplantation. The score correlates inversely with conventional histopathological assessment and correlates with the ACR at 1 year after renal transplantation. Extrapolating from chronic kidney disease, where a decrease in albuminuria is indicative of a response to treatment, the increase in albuminuria could indicate the progression of kidney damage.[39] Utilising RTCA pretransplant correlates with ACR and hence serves as a potential surrogate for eventual proteinuria.

However, prospective multicentre trials are needed to evaluate the score's concordance statistics in a larger cohort, including living donor and DCD kidneys. It is important to demonstrate its applicability in the daily routine and to implement RTCA into already existing DGF-prediction models.[5]

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DISCUSSANTS

Elmi Muller (Cape Town, South Africa):

In transplantation, many difficult decisions regarding organ viability and quality need to be made before the organ is transplanted. Physicians generally rely on clinical markers, such as the protein creatinine ratio and serum creatinine, when assessing the donor organ before transplantation. Many important clinical outcomes are dependent on the quality of the donor organ.

The confocal score's prediction of Delayed Graft Function (DGF) indirectly predicts tubular injury, but the long-term clinical significance of this finding is debatable. Potentially, this methodology could influence clinical decision-making for the patient, such as in the choice of induction agents or the use of calcineurin inhibitors in high-risk recipients. How do the authors see the clinical significance of the scoring system?

The value of scoring kidneys before biopsy should be considered carefully. There is a risk that scoring kidneys before transplantation results in a higher discard rate. This tool should rather focus on the improved allocation of kidneys, in terms of their long-term outcome. Is there a way that the confocal score could be used to improve the allocation of organs?

Last, the confocal score predicted proteinuria in the longer term. Indeed, kidneys with a confocal score lower than 0 showed a higher rate of proteinuria at 3 and 12 months posttransplant. This is important, as proteinuria is an important prognostic indicator for long-term renal function. How will this score be used to predict long-term outcomes in grafts?

Response From Annemarie Weissenbacher (Innsbruck, Austria):

Thank you very much for your questions. Regarding your first question of whether it has clinical significance, I think it would be very helpful to have a marker to predict the occurrence of DGF more precisely. DGF is often debated, as it is multifactorial and might not have the same impact on all organs. However, if we get to know the kidney better, especially in a recipient highly likely to develop DGF, we could adapt our treatment.

We do not have that many options to adapt an immunosuppressive protocol with the clinical drugs available at the moment, and within the pilot trial we applied our current institutional practice. To get a baseline of the value of our RTCA score and achieve the possibility to individualize immunosuppressive treatment in the future, we performed a ROC analysis. The c-statistic for the RTCA score was 0.69. It is not as high as we hoped it would be, but the c-statistic is higher than the one of the clinically used UKKDRI and KDRI scores with c-statistics of 0.63 and 0.62.

With regard to your second question about the organ discard and allocation, we definitely do not want to see our score as a tool increasing the already high discard rate of kidneys even more. This is definitely a disadvantage of scoring systems and the immanent risk of scores with a predictive value. Essentially, we believe that this could go both ways. With kidneys, which look bad on paper and have a higher Remuzzi score, a good RTCA score may suggest little additional damage, and hence, prompt eventual acceptance. In summary, we believe that the RTCA adds an important element to the assessment of kidneys for transplantation and that this would eventually result in better decision-making.

Regarding allocation, it is our wish, of course, that we could influence it and achieve ''individualized'' transplantation. However, by the time we are able to see the result of the RTCA score, the kidney has already been allocated to a recipient. It would be desirable to receive the score earlier, to be able to allocate the best possible organ to the perfect suitable patient.

The other significant advantage of the score might be, as you mentioned in your last question, the prediction of proteinuria, as it is an important and well-known factor influencing 1-year graft survival, and also patient survival after kidney transplantation. If a kidney has a lower RTCA score to start with, we could put the patient under closer observation, monitor proteinuria, optimize blood pressure, lipid metabolism, and so on, and we may also be able to start available treatment earlier.

Norbert Senninger (Münster, Germany):

Thank you. I have a very short question and comment. The comment would be that your clinical implication would also go into nephrology for inherent kidney disease. So, I think that this is very interesting. Please give us an idea of the timeframe required to complete this assessment. When we want to use a marginal organ, we do not have too much time to wait for the result.

Second, for instance, would this be a tool to use in living related donations, to perhaps increase the donor pool? We believe that we have clear ideas on which donors not to use, but if you were to find a good relation and assessment, we could potentially use a marginal organ in living related donors.

Response From Annemarie Weissenbacher (Innsbruck, Austria):

Thank you very much for this important comment. It is not a time-consuming procedure at all: the staining and the analysis with the confocal microscope to get the score takes 30 minutes overall, which is quick. Another advantage of this procedure is the fact that the stains to get to the RTCA score are not toxic, and therefore, exactly the same piece of biopsy can be sent for histological analysis. So, depending on your institution's policy, you do not need to wait hours to get the result.

With regard to your question about living donors, I assume that you would like to have the native biopsy during the assessment of living donors. We do not have experience in this, but it would definitely be an optimal tool for an interesting approach. This would need to be evaluated within a prospective clinical trial. Also, at our institution, we would not perform a native biopsy for a living donor, as you always have to thoroughly consider and discuss the risk versus the benefit. However, it is definitely a good idea, especially if you want to push the boundaries for accepting living donors on a wider basis.

Rutger Ploeg (Oxford, United Kingdom):

I think that this is a very exciting new tool with great potential. We know that, while annoying, DGF can be overcome and function will be restored. Therefore, my question whether the confocal tissue assessment tool has been correlated with GFR of the kidney graft at 1 year?

If I may, I do not think that you should focus on DGF, but rather on the prediction of PNF as Primary Non (and never) Function is devastating followed by graft failure. Patients have to go back on the waiting list, so if you can predict PNF, it would be most helpful.

Last, did you check whether the timing of obtaining the kidney biopsy will affect the predictability of outcome using this tool; in other words is it better to use the postreperfusion biopsy or biopsy obtained at the donor center after flush-out or when inspecting on the back-table immediately before transplantation? Is the predictability of your outcome better in a perfused kidney or cold kidney graft?

Response From Annemarie Weissenbacher (Innsbruck, Austria):

Thank you for your questions. It would be great to predict primary nonfunction, and we had one case with a −3 RTCA score, where the Remuzzi was initially reported as 4 but eventually and most unfortunately revised by the pathologist to a Remuzzi 7 after the transplant has taken place. This was a kidney, which had PNF and was lost in the end. To predict PNF within a trial, it has to be set up as a multicenter trial as the incidence is fortunately significantly lower than the incidence of DGF; however, the graft and patient outcome is knowingly more detrimental.

Regarding the question about the GFR, we could not observe a correlation with the GFR, neither with 3 nor with 12 months GFR. The Remuzzi score did not correlate with it either. The small number of transplants in our pilot trial could well be a factor for this.

To address your last question, it is important to state that I am not able to offer a satisfying answer, as we only have the result of one biopsy, which was always taken after unpacking the kidney during the benching procedure. However, in our experience, the duration of cold ischemia time did not impact the occurrence of DGF and did not correlate with the RTCA score either. According to your question, it would be perfect to gather information of as many biopsies as we can. It would be preferable to compare the result of the perfused kidney in the donor with the result after an undefined duration of cold ischemia time and a postreperfusion biopsy, but I expect we would not get an ethics approval for this type of clinical trial. What would be a future prospect though is the application of our described method in combination with hypo- and/or normothermic kidney preservation especially for extended criteria donors; the hypothesis could be that we see an improvement or change of the RTCA score over time.

Peter A. Lodge (Leeds, United Kingdom):

I have 2 brief questions. What is the applicability of this, in that can this be done in an average kidney transplant center?

Second, what is the clinical relevance, as delayed graft function does not mean that you cannot transplant the kidney?

Response From Annemarie Weissenbacher (Innsbruck, Austria):

Thank you very much. It can be performed at every kidney transplant center, provided that a confocal microscope is available. In regard to your second question, I completely agree with you – the potential occurrence of DGF does not mean and must not mean that the kidney should not be transplanted. However, what we know and what we have to consider is the fact that it is still an important factor impacting graft as well as patient survival, and it needs to be avoided or its effects minimized exactly for this reason. There is an excellent study, which I think was published in 2016 in *Kidney International*, which observed in a cohort of about 28,000 patients that the ones with DGF had a higher mortality risk immediately after transplantation, when compared with the patients remaining on the waiting list. Of course, we know that dialysis is more detrimental in the long run, and another result of this study shows that patients with DGF will gain similar mortality risk as patients with initial kidney function with time. However, if we are able to treat these patients with DGF better or put them under closer surveillance, we could be able to antagonize the detrimental effects of DGF.