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# Novel Ex-Vivo Thrombolytic Reconditioning of Kidneys Retrieved 4 to 5 Hours After Circulatory Death

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**Background.** Due to organ shortage, many patients do not receive donor organs. The present novel thrombolytic technique utilizes organs from donors with uncontrolled donation after circulatory deaths (uDCD), with up to 4–5 h warm ischemia, without advanced cardiopulmonary resuscitation (aCPR) or extracorporeal circulation (EC) after death. **Methods.** The study group of pigs (n = 21) underwent simulated circulatory death. After 2 h, an ice slush was inserted into the abdomen. Kidneys were retrieved 4.5 h after death. Lys-plasminogen, antithrombin-III (ATIII), and alteplase (tPA) were injected through the renal arteries on the back table. Subsequent ex vivo perfusion at 15 °C was continued for 3 h, followed by 3 h with red blood cells (RBCs) at 32 °C. Perfusion outcome and histology were compared between uDCD kidneys, receiving no thrombolytic treatment (n = 8), and live donor kidneys (n = 7). The study kidneys were then transplanted into pigs as autologous grafts with a single functioning autologous kidney as the only renal support. uDCD control pigs (n = 8), receiving no ex vivo perfusion, served as controls. **Results.** Vascular resistance decreased to <200 mmHg/mL/min ( $P < 0.0023$ ) and arterial flow increased to >100 mL/100 g/min ( $P < 0.00019$ ) compared to controls. In total 13/21 study pigs survived for >10 days, while all uDCD control pigs died. Histology was preserved after reconditioning, and the creatinine level after 10 days was next to normal. **Conclusions.** Kidneys from extended uDCD, not receiving aCPR/EC, can be salvaged using thrombolytic treatment to remove fibrin thrombi while preserving histology and enabling transplantation with a clinically acceptable early function.

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## INTRODUCTION

The lack of donor organs limits the use of transplantation as a treatment for end-stage renal disease (ESRD), and the estimated global need for organ donation is met to <10%.<sup>1</sup>

Donations after brain death (DBD) are the primary source of donor organs (77.3 %).<sup>2</sup> In Sweden, 92 000 people died during 2018, but <200 (0.22 %) deaths resulted in organ donation. Living donors are another essential source of donor organs, but the donor pool remains insufficient

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M.O. is the inventor of several patents within the field of transplantation. The other authors declare no conflicts of interest.

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

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the performance of the research, D.B. participated in the performance of the research, collection and analysis of the data, G.T. in performance of the research, and analyzed the data. G.U.P. performed the research and collected the data. M.J. participated in the data analysis. J.M.S. participated in writing the article and analyzing the data. All authors provided critical feedback and helped shape the research. D.A., G.T., and G.U.P. took equal and the main parts of the work. Supplemental Visual Abstract; <http://links.lww.com/TP/C336>.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site ([www.transplantjournal.com](http://www.transplantjournal.com)).

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to meet the required number of patients. Further initiatives employing xeno-transplantation<sup>3</sup> and regenerative medicine<sup>4,5</sup> have not yet resulted in a larger donor pool.

If organs from patients dying outside the hospital (OHD) could be harnessed for transplantation, there would be no donor shortage. Organs from circulatory death patients include controlled donations after circulatory death (cDCD) and uncontrolled donations after circulatory death (uDCD).<sup>5-14</sup> The proportion of donations after circulatory death (DCD) has increased substantially, amounting to 20% of organs from deceased patients in Europe,<sup>2</sup> however, two obstacles remain largely unresolved for uDCD, meaning that this approach tends to be an exception rather than a rule.

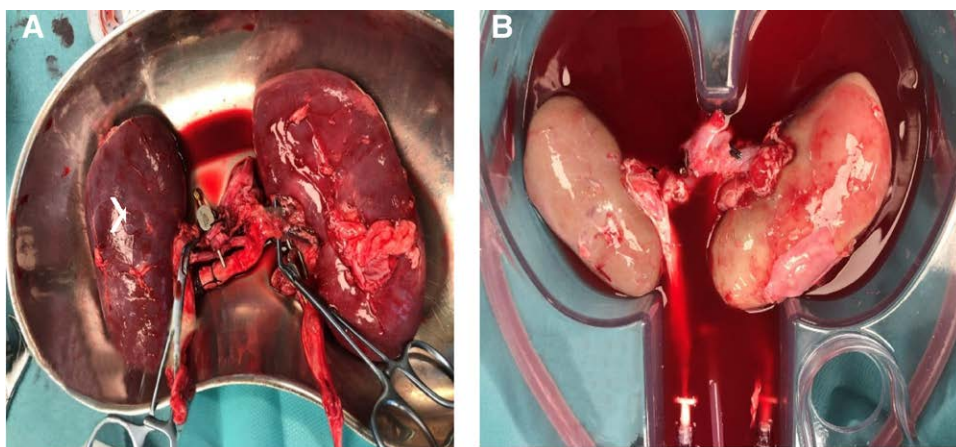
One major problem is the prolonged warm ischemia time (WIT), which begins when blood circulation ceases until the point at which donor organs can be cooled and preserved. Typically, advanced cardiopulmonary resuscitation (aCPR), followed by either manual or machine chest compression and/or extracorporeal circulation (EC), must be initiated for uDCD donors to be accepted as potential donors. Despite these measures, kidney recipients of uDCD donors typically present with acute tubular necrosis (ATN), observed as delayed graft function (DGF).<sup>6-14</sup> Acute rejection with differential diagnostic difficulties, vascular thrombosis, and prolonged hospitalization are routinely observed in these recipients.<sup>8,12,15</sup>

Families of the deceased need time to provide donor consent after confirmation of a close relative's death. The elapsed time between death and organ retrieval has effectively stopped any meaningful attempt to use organs from uDCD in countries that lack laws that allow rapid intervention to preserve organ function due to WIT, raising concerns about the division between treatment and organ procurement/preservation.<sup>16-18</sup> Regardless of whether there is a legal possibility to intervene early or not, a technology that would allow a longer period from cardiac arrest and subsequent declaration of death, to the retrieval of the organs, would greatly simplify the process. This is contingent on the mitigation effects of extended WIT. Continuing aCPR/EC, while waiting for consent and the transplant team would not be needed, and this obstacle to using DCD donors could be removed.

Furthermore, with a longer period and clear separation between treatment and organ procurement/preservation, concerns from the public and the profession would probably be less. Even better, if the frequency of DGF could be improved by addressing the physiological changes caused by ischemia-reperfusion injury (I/R-I), despite the longer period for retrieval, many of the economic hurdles of the uDCD process could be overcome and made more widely used. Hansen et al.,<sup>19</sup> found fibrin thrombi in 3% of DBD kidneys in a retrospective study and found this to be a risk factor for reduced renal function at 1-y post-transplantation. These findings are further supported by a recent study by DiRito et al.,<sup>20</sup> who examined the effect of plasminogen and tissue plasmin activator (tPA) on marginal kidneys in their ex vivo normothermic machine perfusion (NMP) model, found that clearing fibrin from the capillaries in the tubuli resulted in a decreased vascular resistance. Plasminogen occurs naturally in human plasma as Glu-plasminogen and is converted naturally to the much more effective Lys-plasminogen after first binding to fibrin.<sup>21</sup> By adding tPA all three components bind, fibrin converts to fibrinogen and plasminogen to plasmin, thus dissolving the clot.<sup>21</sup> There is a risk of rethrombosis due to the activated endothelium and platelets after dissolving the clot, despite the presence of naturally occurring antithrombin-III (ATIII). We used a direct thrombin inhibitor (argatroban)<sup>22</sup> and a platelet inhibitor (abciximab)<sup>23</sup> to prevent this.

To differentiate between allogeneic response and ischemia-reperfusion reactions we designed an autotransplantation model for uDCD, as described in Figure 1 and in a previous publication.<sup>24</sup> The short period of 30 min as an "allogeneic" graft in the donor did not result in rejection (unpublished data), showing that we primarily studied I/R-I.

The aim of this study was to use a new thrombolytic treatment procedure utilizing Lys-plasminogen and tPA (alteplase), to address the physiological changes caused by ischemia. After allowing ex vivo reconditioning of donor kidneys from pigs 4.5 h after circulatory arrest we intend to show that the function can be retained using the newly suggested treatment.



**FIGURE 1.** (A) Retrieved uDCD kidneys en-bloc after 4–5 h ischemia. The kidneys will be reconditioned and one of the kidneys will be transplanted to a recipient. (B) Kidneys after completed reconditioning, ready for transplantation. uDCD, uncontrolled donation after circulatory death.

## MATERIALS AND METHODS

### Animals and Study Design

Swedish domestic pigs ( $n = 73$ ) weighing 30–50 kg were procured for the study. The study design is shown in Table 1. An autotransplantation model for uDCD was developed, to avoid allogeneic reactions. See Materials and Methods S1, SDC <http://links.lww.com/TP/C335> for additional information.

### Ethical Permit

The Regional Animal Experiment Ethics Committee approved the study (Dnr 5.8.18-13977/2018; Dnr 5.8.18-09474/2019).

### Anesthesia and Experimental Equipment

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Blood Grouping

See Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Red Blood Cell Washing

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Ex Vivo Perfusion Device

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

**TABLE 1.**

**Groups included in perfusion and in functional studies (transplantation/creatinine)**

Group	Recipients (n)	Donors (n)	Treatment	Total (n)
I	21	21	uDCD, perfused with base solution and given thrombolysis, later transplanted	42
II	N/A	8	Kidney from healthy pigs, retrieved as a live donor kidney, perfused with base solution without thrombolysis, not transplanted	8
III	N/A	7	Kidney from uDCD pigs, perfused with base solution without thrombolysis, not transplanted	7
IV	8	8	uDCD, receiving no perfusion and no thrombolytic treatment, transplanted after retrieval	16
Sum	29	44		73

I. One kidney was transferred from the recipient ( $n = 21$ ) to a pig and subsequently turned into an uncontrolled donation after circulatory deaths (uDCD) donor ( $n = 21$ ). II. After reconditioning, the same kidney was transplanted into the recipient. Kidneys retrieved as live-donor kidneys. III. Kidneys were retrieved as uDCD kidneys, receiving no thrombolytic treatment, but perfused with UGLK-S. IV. Kidneys from an uDCD donor pig. The kidney did not receive thrombolytic treatment and was only perfused with 2–300 mL IGL-1 on the back table before transplantation. Groups I–III: used for perfusion studies.

Groups I–IV: used for immunohistochemistry/histology studies.

Group I: used for transmission electron microscopy studies.

Groups I and IV: used for early functional studies (transplantation survival/creatinine).

uDCD, uncontrolled donation after circulatory deaths.

### DCD Protocol

Donor pigs were anesthetized and maintained under normoventilation. Blood gases, blood pressure, weight, and length of the pigs were recorded. The ventilator was turned off, and the time of asystole, occurring within 15 min for all pigs was recorded as the time of death. Pigs were left at room temperature for 2 h before a small midline incision was made, and an ice slush was poured into the abdomen. Temperature probes were placed in the liver and right flank muscles, close to the right kidney (Table 2). Each pig was left for two additional hours before both kidneys were removed en bloc with the caval vein and the aorta. The mean time from circulatory death until the organs were retrieved was 4 h 30 min (range, 260–280 min).

### Perfusion Procedure

From each animal in the study group ( $n = 21$ ), the harvested kidneys were placed in a bowl on an ice bed on a back table (Figure 1A). The arteries of both kidneys were injected with UGL-K-A (30 U of Lys-plasminogen; 600 U ATIII) in a volume of 6–7.5 mL per kidney (group I). The renal vein and artery ends were both closed, preventing the loss of solution from the kidneys. After 15 min, the arteries were opened while maintaining vein closure to allow injection of UGL-K-B (6 mg alteplase; 600 U ATIII) in a volume equal to UGL-K-A. The arteries were clamped again for 15 min. Plastic adapters were mounted on the aorta to monitor pressure, flow, and resistance during kidney perfusion. Baby-feeding catheters were inserted into the ureters. Both kidneys were then moved to a custom-built ex vivo perfusion device. The aorta was connected to the perfusion pump, the pressure transducers were calibrated, and 2 L of oxygenated perfusion solution (UGL-K-S) was adjusted to 24 °C (Table 3). Perfusion was initiated at 20 mmHg with a cell-free oxygenated perfusate for 3 h. The pressure was increased by 5 mmHg every 5 min up to 70–85 mmHg, or until both kidneys were completely cleared of dark spots. The temperature was then lowered to 15 °C, and the pressure was reduced to 20 mmHg while observing and recording resistance and flow, as well as urinary output and blood gases. Washed red blood cells (RBCs) were mixed with UGL-W (argatroban; abciximab; ATIII), added to the perfusion solution after increasing the temperature to 28 °C, and allowed to perfuse with a separate pump device containing adsorbers for endotoxins and chemokines/cytokines, followed by further increasing the temperature to 32 °C and pressure to 30 mmHg. The perfusion was continued for 3 h, observing and registering resistance and flow, as well as urinary output and blood gases. At the end of perfusion, the pressure was decreased to 20 mmHg, and

**TABLE 2.**

**Temperature change in the liver and in the right flank after circulatory death**

	Liver probe	Flank probe
Temp at start (°C) (Range) $n = 8$	36.6 (36.0–37.1)	36.6 (36.0–37.1)
Temp after 2 h (°C) (Range) $n = 7$	35.0 (34.2–36.3)	34.8 (33.2–36.4)
Temp after 4 h (°C) (Range) $n = 7$	13.5 (6.4–19.8)	13.4 (7.8–19.6)

The temperature change of the uncontrolled donation after circulatory death donors was measured with a probe in the liver and one probe in the flank region, close to the kidney. Values are expressed as the median temperature with range.

**TABLE 3.**  
Composition of the perfusion solution (UGL-K-S)

Component	Conc in mM/L
Calcium chloride	4.58
Adenine	5
Dextrose	5.55
Magnesium gluconate	2.73
Potassium phosphate	5
Sodium gluconate	50
L-Leucine	0.396
Potassium gluconate	17
L-Arginine monohydro-chloride	0.603
L-Glutamine	2
D-Calcium pantothenate (vitamin B5)	4.20
Choline chloride	0.007
Folic acid	0.002
I-inositol	0.111
Niacinamide	0.008
Pyridoxine monohydrochloride	0.005
Riboflavin (vitamin B2)	0.0003
Thiamine monohydrochloride (vitamin B1)	0.003
Ribose	5
Sterile water	
Albumin 57 g	
Novorapid 20 U	

the temperature was decreased to 15 °C before retrieving the kidneys for transplantation (Figure 1B) as autologous transplants.<sup>24</sup> The study protocol is shown in Table 1.

### Transplant Study

A comparison between the study protocol and untreated uDCD was performed using transplantation. Since no dialysis was administered, a survival of 10 d or more was considered a significant outcome.

### Blood Gas and Creatinine Analysis

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Histology

Representative histological materials from the four groups described in Table 1 were analyzed in this study. See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335> section.

### Immunohistochemistry

To confirm the presence of fibrin plugs in the renal capillaries of kidneys not treated with thrombolytic drugs, before and after machine perfusion, immunohistochemistry was performed. See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335> section.

### Arterial Flow

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Transmission Electron Microscopy

See the Materials and Methods S1, SDC, <http://links.lww.com/TP/C335>.

### Statistics

WinSTAT for Excel (Robert Fitch software) was used for data analysis. The Mann-Whitney U test was used for comparisons between groups, and the Wilcoxon signed-rank test was used for comparisons within groups. Values are presented as mean or median with range. OriginLab was used for the graphical presentation of scientific data.

## RESULTS

### Ex Vivo Flow and Resistance During Perfusion

Vascular resistance and arterial flow from autologous transplanted kidneys during the reconditioning phase are shown in Figure 2 A and B. Resistance ( $P < 0.0023$ ) and flow ( $P < 0.00019$ ) were significantly better than those in the nonthrombolysis-treated control kidneys. There was no significant difference between the pigs surviving for 10 days or more compared with the pigs surviving for <7 days (Figure 3 A and B). Similar values for resistance and flow were also achieved for the study group kidneys compared to healthy untreated control kidneys, although the latter group was only followed during cold perfusion. The arterial flow was corrected for kidney weight after retrieval and indexed as mL/100 g kidney tissue/min. A level of vascular resistance below 200 mmHg \* min/L and an arterial flow above 100 mL/100 g/min consistently resulted in good arterial flow after reperfusion.

### Arterial Flow

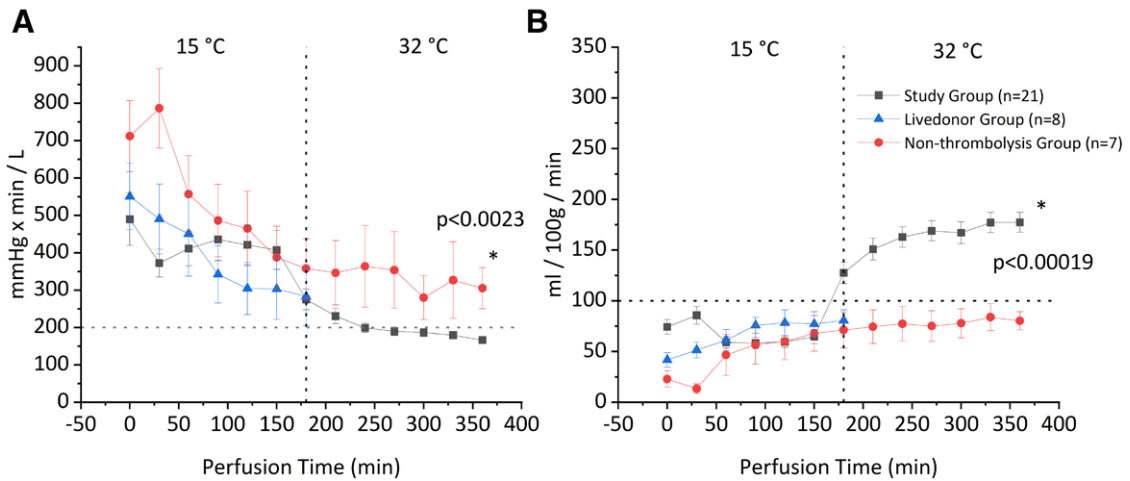
Arterial flows of the study group, and the uDCD control group—before intervention (measured in 14 pigs), at reperfusion, and 90 min after reperfusion—can be seen in Figure 4 A–C. Arterial flow at reperfusion was significantly better in the study group ( $P < 0.000039$ ), however, 90 min after reperfusion there was no significant difference between the groups.

### Renal Function and Survival

Pigs subjected to the study protocol were transplanted into the native pig as an autologous graft, as recently described.<sup>24</sup> Thirteen of the 21 transplanted pigs survived 10 d or more, with the reconditioned kidney as the only renal support. Initial complications due to the learning curve of the autologous transplantation procedure resulted in high initial mortality (Table 4). Creatinine was measured at reperfusion in anesthetized pigs, 90 min after reperfusion, and after 10 d when possible (Figure 5). This function was comparable to that observed in untreated animals with a single kidney. No statistically significant differences in creatinine levels were observed at 90 min or 10 d. In comparison, the uDCD control group showed normal creatinine levels at 90 min after reperfusion; however, only two pigs survived to day 5 (Figure 6), with creatinine levels of over 1000 μmol/L at the time of death.

### Histological Changes

Changes in the different groups described in Table 1 are shown in Figure 7. All tissues were also stained with Masson's trichrome which failed to demonstrate fuchsinophilic thrombi in any of the cases.



**FIGURE 2.** (A) shows the changes in vascular resistance and (B) the arterial flow in kidneys perfused using the study group I protocol (n = 21), in black squares with solid lines. The flow and resistance were compared to a group of healthy normal kidneys, retrieved as live donor kidneys, labeled as group II (n = 8) in blue solid line and triangles perfused with only perfusion solution without thrombolytic drugs, and to a group of kidneys from uDCD without thrombolytic drugs (n = 7), but reconditioned with perfusion solution, labeled group III (n = 7) in red solid line and circles. The dotted horizontal black line marks the 200 mmHg \* min/L limit of vascular resistance in (A) and the 100 mL/100g/min limit of arterial flow in (B). The dotted vertical black line marks the shift in temperature from 15 °C to 32 °C, and the increase in pressure from 20 to 30 mmHg. Group I had a significantly lower vascular resistance ( $P < 0.0023$ ) and higher arterial flow ( $P < 0.000019$ ) than group III at the end of the evaluation, using the Mann-Whitney U test. uDCD, uncontrolled donation after circulatory death.

**Immunohistochemistry**

Kidneys from uDCD before treatment showed the presence of fibrin/fibrinogen (Figure 8 A.1 and A2), changes that largely disappeared after thrombolytic treatment and perfusion (Figure 8 A3–A6). Kidneys retrieved from live donors (group II) showed no signs of fibrin/fibrinogen before perfusion (Figure 8 B1 and B2), but activity appeared after cold perfusion (Figure 8 B3 and B4) and remained during perfusion with RBC (Figure 8 B5 and B6). uDCD kidneys treated with the base solution and no thrombolysis also had fibrin/fibrinogen activity at the end of the evaluation (Figure 8 C1 and C2), and uDCD kidneys transplanted without perfusion and thrombolysis had signs of fibrin/fibrinogen remaining on day 5 after

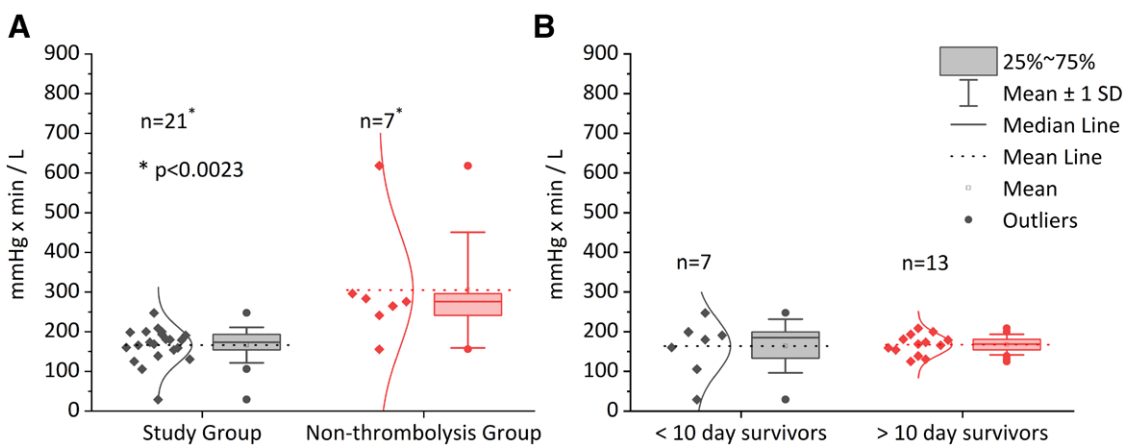
transplantation when the pigs were sacrificed due to kidney failure (Figure 8 D1 and D2).

**Transmission Electron Microscopy Images**

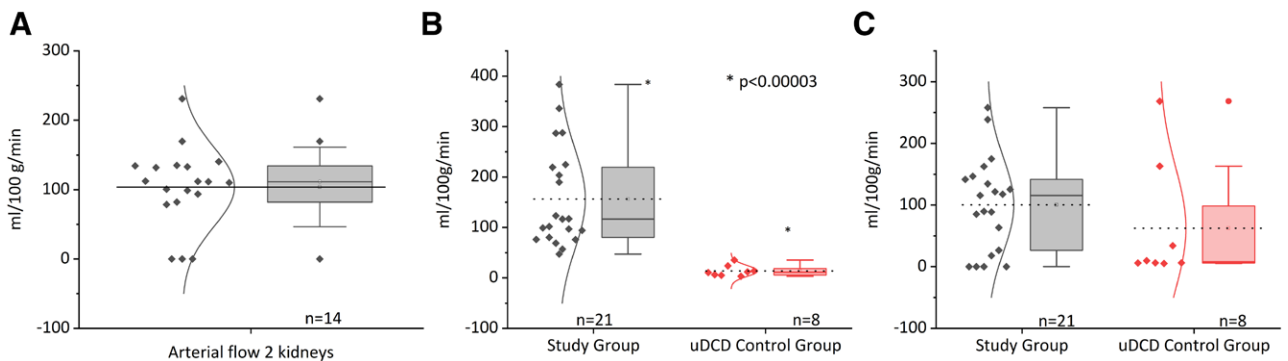
The transmission electron microscopy (TEM) images from biopsies taken at different time points during the perfusion revealed cleaner capillaries after, compared to before, the perfusion treatment (Figure 9 A–E).

**DISCUSSION**

In this article, we report a novel and clinically relevant method of extended uDCD, using thrombolytic treatment, with Lys-plasminogen and alteplase, and ex vivo perfusion in pigs. The model allows for >4 h of ischemia



**FIGURE 3.** Box chart of vascular resistance in mmHg \* min/L after 360 min of ex-vivo perfusion. The box shows 25%–75% percentile range of datapoints. Diamonds, in black or red, show individual datapoints, round symbols show outliers. Whiskers show 1 SD, mean value are marked as a dotted line, median value as a solid line. A normal distribution line is plotted beside the data points. In (A) the study group (group I), in black, is compared to the nonthrombolysis group (group III), in red. Group I had a significant lower vascular resistance ( $P < 0.0023$ ) and higher arterial flow ( $P < 0.000019$ ) than group III at the end of evaluation, using Mann-Whitney U. In (B), a post hoc analysis was performed to study any difference between pigs that had a functioning kidney >10 d (n = 13) vs <7 d (n = 8). No significant difference in vascular resistance could be seen.



**FIGURE 4.** Box chart of arterial flow in anesthetized pigs. The box shows 25%–75% percentile range of datapoints. Diamonds, in black or red, show individual datapoints, round symbols show outliers. Whiskers show 1 SD, mean value are marked as a dotted line, median value as a solid line. A normal distribution line is plotted beside the data points. In (A) arterial flow was measured in anesthetized pigs, before any intervention of the kidney in 14 pigs, in (B) arterial flow was measured at reperfusion of study group ( $n = 21$ ) kidneys in black and uDCD control ( $n = 8$ ) group in red. In (C) arterial flow 90 min after reperfusion of study group ( $n = 21$ ) kidneys in black and uDCD control ( $n = 8$ ) group kidneys in red. Flow was significantly better in the study group vs the uDCD control group at reperfusion ( $P < 0.00003$ ), but not significantly different 90 min after reperfusion, as measured with Mann-Whitney U-test. uDCD, uncontrolled donation after circulatory deaths.

after circulatory arrest, with surprisingly few histological changes after reconditioning and preserved function after 10 d.

The addition of slushed ice significantly reduced the temperature of the viscera within the abdominal cavity (Table 2), although most clinicians would consider that the internal organs were maintained at a temperature higher than would be considered typical of “cold” ischemia. This is especially so because the organs are not cleared of blood with cold solution, thus achieving only surface cooling. The organs were also not well oxygenated. Using an *ex vivo* reconditioning protocol, we demonstrated the feasibility of reconditioning these donor organs for subsequent transplantation, previously believed to be beyond salvage.

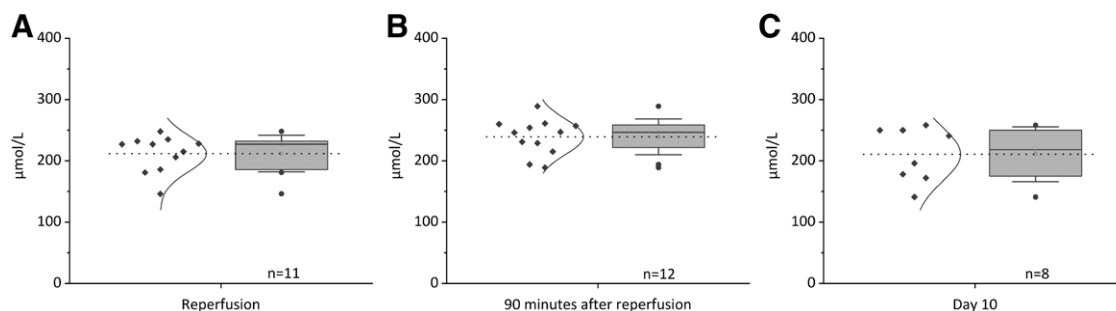
A key consideration in the present extended uDCD model’s design was to manage a typical day-to-day clinical scenario more effectively. In a clinical setting, aCPR is continued during efforts to obtain consent in an ethically acceptable manner, resulting in a shortage of time. In contrast, our protocol allows an extended time window of 4 h, without the need for EC or manual compression, which should be sufficient to manage consent and retrieve viable donor organs in an ethical process. Furthermore, our approach may offset the physiological hurdles associated with such a dramatic increase in warm ischemia time. The present study proves that it is feasible to salvage organs subjected to prolonged warm ischemia for transplantation using well-established tools to prevent irreversible ischemic damage to the organ.

In cDCD Maastricht III donors,<sup>25</sup> use at least 5 min of “stay-off/no-touch” to ensure that the cardiac failure is irreversible, and at the same time, there will be no recovery of the brain injury following the circulatory arrest. In patients undergoing cardiopulmonary resuscitation, some individuals develop brain edema, which may progress to brain death.<sup>26</sup> In organs subjected to warm ischemia, the function often declines reversibly after reperfusion. Our knowledge of the various mechanisms involved in I/R-I is vast. However, to date, no single procedure has been described that allows successful transplantation of organs exposed to prolonged warm ischemic insults, without the use of aCPR and/or EC. Using these technologies, on the other hand, has resulted in kidney, lung, liver, and even heart,<sup>2</sup> both after cDCD and after uDCD. As already mentioned, organs from DCD donors will result in an increased risk of DGF due to ATN. Although uDCD programs are in use, organs from these donors are rare, with an estimated frequency of only 2.4% of all transplanted DCD organs,<sup>2</sup> and good long-term results have been reported.<sup>27</sup> Centers with a higher proportion of uDCD also have a significantly lower graft survival compared to standard criteria brain-dead donors.<sup>28</sup> Currently, the uDCD donor is expensive, requires vast resources, and is usually performed only in large university hospitals.<sup>2</sup> A simplified and improved process, would increase the number of potential donors, even with conservative estimates, by an order of magnitude in Sweden.<sup>29</sup>

It is well known in cardiac patients with a circulatory arrest that fibrin thrombi are formed without the simultaneous activation of fibrinolysis.<sup>30–33</sup> Rapid intervention and administration of thrombolytic agents via coronary angio-catheters limit the ischemic effects of cardiovascular insult and can be life-saving. These healing processes left to the patient’s own plasma thrombolysis system would require several days.<sup>32,34</sup> Fibrin thrombi have also been reported in kidneys from donors with disseminated intravascular coagulation and naturally in 3%–9% of organs from DBD.<sup>19,35–37</sup> Transplantation often results in DGF in these kidneys,<sup>2,6–14,35,36</sup> although a study from the Mayo Clinic, despite a significant difference in vascular

**TABLE 4.**  
**Early complications after autotransplantation**

Complication	Number	Comments
Artery thromboses/rotation	3	Day 0, 0, 0
Bleeding from vein anastomosis	1	Day 0
Air embolus	1	Day 0
Asphyxia	1	Day 2
Anesthesia complications	2	Day 4, 6
Total	8	

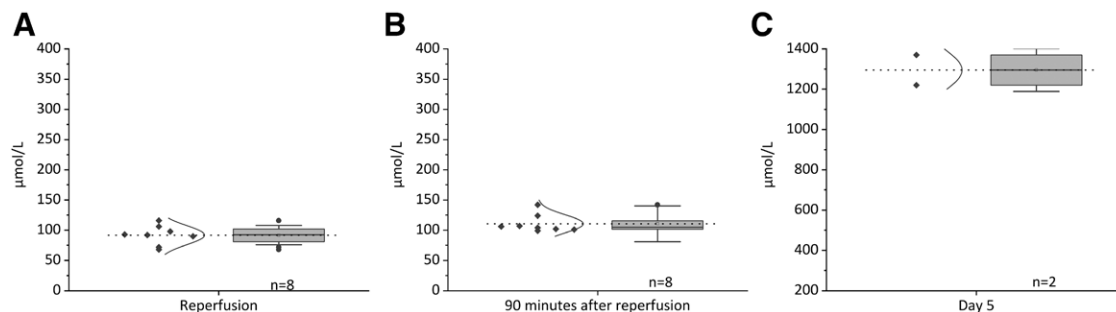


**FIGURE 5.** Box chart of creatinine in transplanted study group (I) pigs. The box shows 25%–75% percentile range of datapoints. Diamonds, in black or red, show individual datapoints, round symbols show outliers. Whiskers show 1 SD, mean value are marked as a dotted line, median value as a solid line. A normal distribution line is plotted beside the data points. In (A) creatinine was measured in anesthetized pigs, before any intervention of the kidney, in (B) creatinine was measured at 90 min after reperfusion of the study group (n = 21) kidneys. In (C) creatinine was measured at 10 d in the study group (n = 8) kidneys creatinine was measured in 8/13 pigs surviving days or longer.

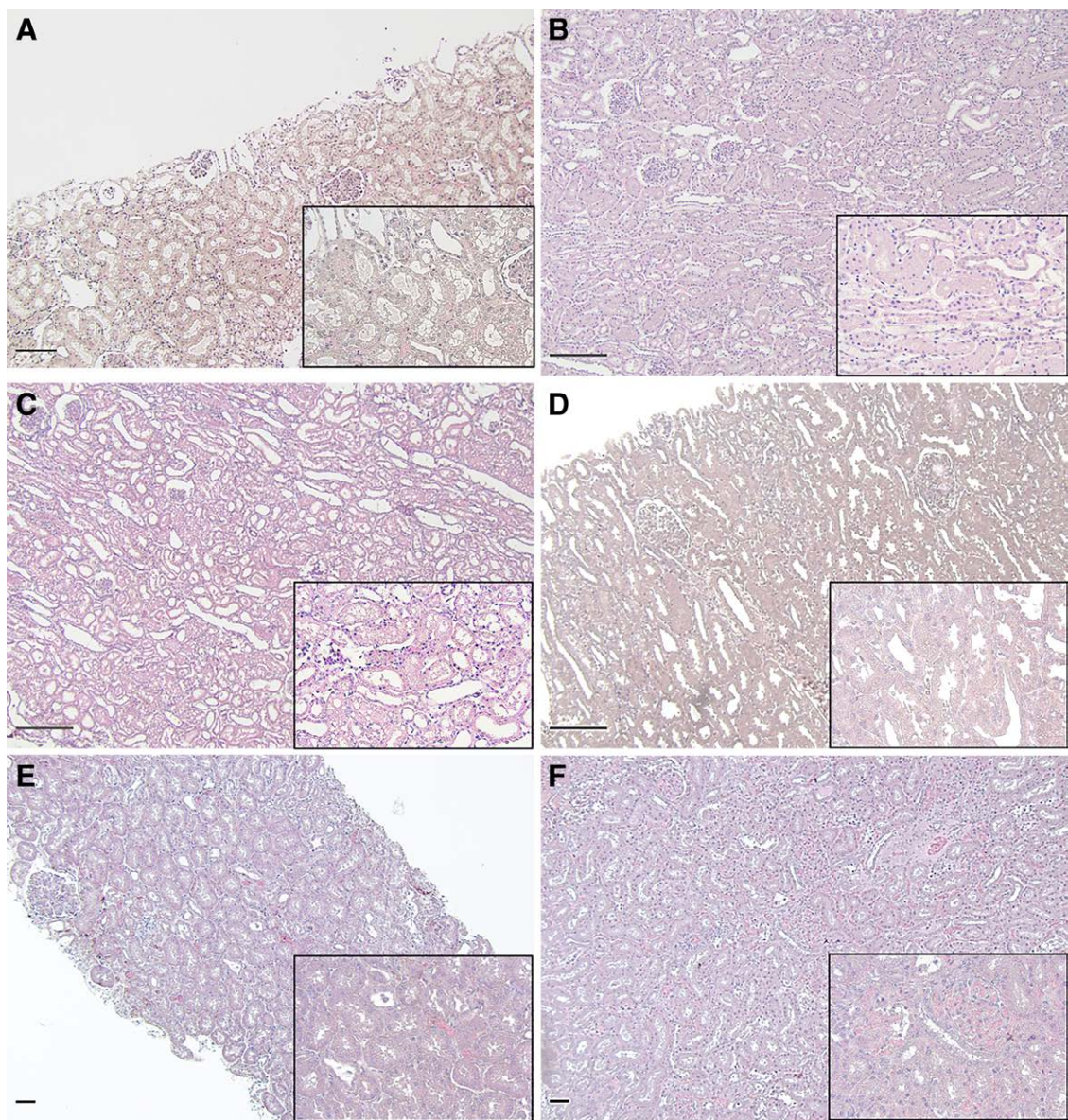
resistance and arterial flow at evaluation, no significant difference in DGF was observed, although the control material had 39% and the study group had 49% DGF, that is, higher levels than more commonly reported.<sup>37</sup> Based on these and other studies in experimental animals and humans<sup>21,38–40</sup> as well as our early investigational studies, we selected a combination of Lys-plasminogen and tPA for perfusion and reconditioning of uDCD kidneys. Control uDCD kidneys not treated with Lys-plasminogen frequently appeared dark during perfusion, and sometimes with large black confluent areas suggesting necrosis during and after perfusion. Renal resistance was also high in the kidneys. In contrast, kidneys in the study group receiving Lys-plasminogen and thrombolytic treatment had either no dark areas or at most three small dark spots at the end of the evaluation and showed significantly improved renal flow and renal resistance during perfusion of the study kidneys (Figure 2). In the immunohistochemistry analysis, we demonstrated the presence of fibrin/fibrinogen activity after the uDCD kidneys had been retrieved, changes that disappeared after Lys-plasminogen, tPA, and perfusion. Interestingly, live donor kidneys, without initial signs of fibrin/fibrinogen, showed signs of activity after perfusion without thrombolysis. Furthermore, the activity was clearly visible in kidneys receiving perfusion with just base solution, as well as in kidneys receiving no perfusion or thrombolysis.

The importance of these findings needs further studies. Furthermore, high-resolution TEM showed aggregates of RBCs in the microvasculature of the uDCD biopsies. After treatment with ATIII, tPA, and Lys-plasminogen, most of the capillaries in the biopsies, taken after the cold perfusion and even more so at the end of the RBC phase, appeared free of RBC aggregates in the present study, similar to controls.

To the best of our knowledge, there is no information on fibrin thrombi from uDCD kidneys with an extended time from circulatory stop to retrieval in the literature, although a recent study by DiRito demonstrated microvascular fibrin thrombi in the tubular system<sup>20</sup> of marginal human kidneys, which is similar to what we see in our study, and Sánchez-Fructuoso<sup>7</sup> observed fibrin thrombi in uDCD with primary non function. DiRito cleared fibrin thrombi with plasminogen and tPA during NMP.<sup>20</sup> The “clearing” effect is similar to what we found in our uDCD model. Platelet aggregation from the tubing system may contribute to fibrin clot formation. This can be avoided using heparin-coated tubing.<sup>41</sup> Another factor that may contribute to decreased microcapillary circulation is low levels of ATIII, which may induce heparin resistance and a pro-thrombotic state.<sup>42</sup> Since most ex vivo perfusion systems do not use ATIII, the effect of heparin might be hampered because heparin exerts its main action of thrombin inhibition by binding



**FIGURE 6.** Box chart of creatinine in anesthetized pigs receiving uDCD control kidneys, without any perfusion or thrombolytic treatment (group IV). The box shows 25%–75% percentile range of datapoints. Diamonds, in black, show individual datapoints, round symbols show outliers. Whiskers show 1 SD, mean value are marked as a dotted line, median value as a solid line. A normal distribution line is plotted beside the data points. In (A) creatinine was measured in anesthetized pigs, before any intervention of the kidney; in (B) creatinine was measured 90 min after reperfusion of the uDCD control group (n = 8) kidneys; and in (C) creatinine was measured in the two uDCD control group (n = 2) kidneys surviving 5 d after transplantation. None of the eight animals survived beyond 5 d. uDCD, uncontrolled donation after circulatory deaths.



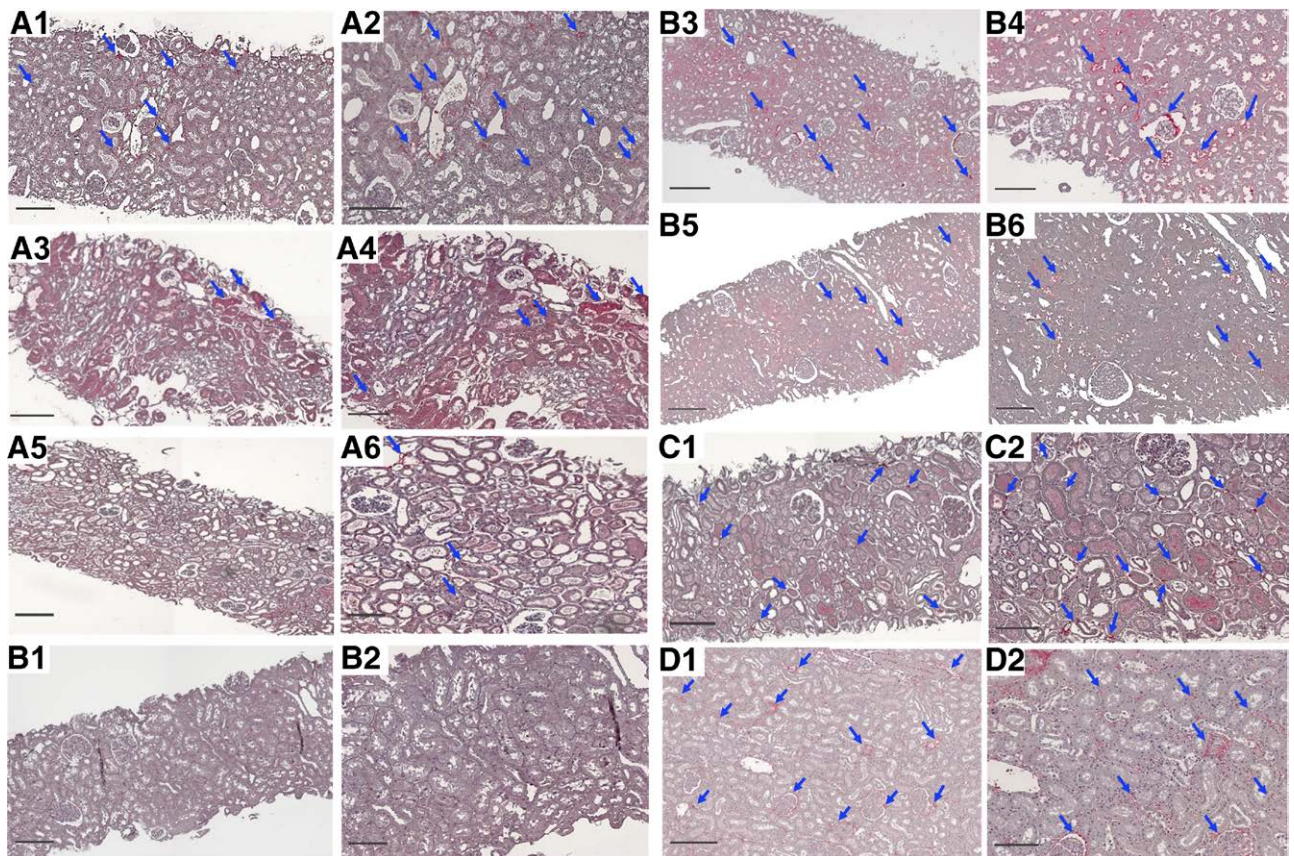
**FIGURE 7.** Evaluation of the kidney histology of the study groups. In (A) histology of kidneys from the study group following ischemia but before reconditioning. Signs of tubular injury can be seen as cellular cytoplasmic vacuolization, epithelial simplification and sloughing of apical membranes into the lumen. In (B) and (C) the postperfusion histology is shown. Again, signs of tubular injury are seen as indicated above, but with less observable apical membrane remnants in the lumen of the tubules. In (D) the histology is shown in kidneys from healthy pigs, retrieved as live donor kidneys, after perfusion with base solution without thrombolytic factors added. The histological changes are similar to those described above. In (E) histology of uDCD kidneys, perfused with base solution and no thrombolytic treatment. Acute tubular injury is seen with simplification, vacuolization, and sloughing apical membranes. In (F) histology from uDCD kidneys subjected to ischemia followed by immediate transplantation without perfusion. Signs of tubular injury was seen with vacuolization and apical sloughing, in two cases this was severe and with distinct interstitial edema. No changes were seen in glomeruli or vessels. Scale bars = 200  $\mu$ m. Inserts shows higher magnification. Stained by Hematoxylin/Eosin. uDCD, uncontrolled donation after circulatory deaths.

to both thrombin and ATIII. No access to ATIII would be similar to the use of tPA alone instead of in combination with plasminogen. The use of argatroban and abciximab in addition to ATIII allowed perfusion of the entire kidney circulatory bed. Adequate perfusion was visually reflected by the kidney surface without evidence of perfusion defects, constant low resistance during perfusion, and high flow rates and gradually removed platelet/RBC aggregates in the TEM imaging.

The steadily decreased resistance during the cold and warm perfusion phases was dependent on oxygen. In some experiments, oxygen was omitted or delayed, with less

favorable outcomes (data not shown). Recent studies have concluded that oxygenated perfusion is superior to a non-oxygenated perfusion solution,<sup>43–45</sup> which has previously been difficult to prove.<sup>46</sup> Perfusion at low temperatures has the advantage that tissue oxygenation occurs via physically dissolved oxygen. Increased temperatures require an oxygen carrier to deliver sufficient oxygen to the tissue. We deliberately chose a temperature of 12–15 °C. This temperature range is unlikely to cause rigidity of lipids in the cellular membrane, which could potentially harm the endothelium during increases in perfusion pressure and flow through the perfused organ.<sup>47</sup>





**FIGURE 8.** Fibrin(ogen) presence in kidneys treated or not treated with fibrinolytic drugs (immunohistochemistry) (A.1) and (A.2) represent uDCD kidney biopsies taken immediately after 4.5h of death. In (A.3 and A.4) and (A.5 and B.6) biopsies collected after cold perfusion and the end of machine perfusion respectively, from uDCD kidneys that were treated with Lys-plasminogen, alteplase and anti-thrombin III (group 1). In (B.1) and (B.2) biopsies collected before machine perfusion of live donor kidneys (group 2). In (B.3) and (B.4) biopsies from live donor kidneys after cold perfusion (group 2), whereas (B.5) and (B.6) show biopsies at the end of machine perfusion of the same group. In (C.1) and (C.2) biopsies collected at end of machine perfusion of uDCD kidneys after 4.5 h of death, which were not treated with thrombolytic drugs but were machine perfused (group 3). In (D.1) and (D.2) uDCD kidneys after 4.5 h of death that were transplanted without thrombolytic treatment or machine perfusion (group 4). Blue arrows show fibrin(ogen). All images on the right are 20× images of images represented on the left at 10×. uDCD, uncontrolled donation after circulatory deaths.

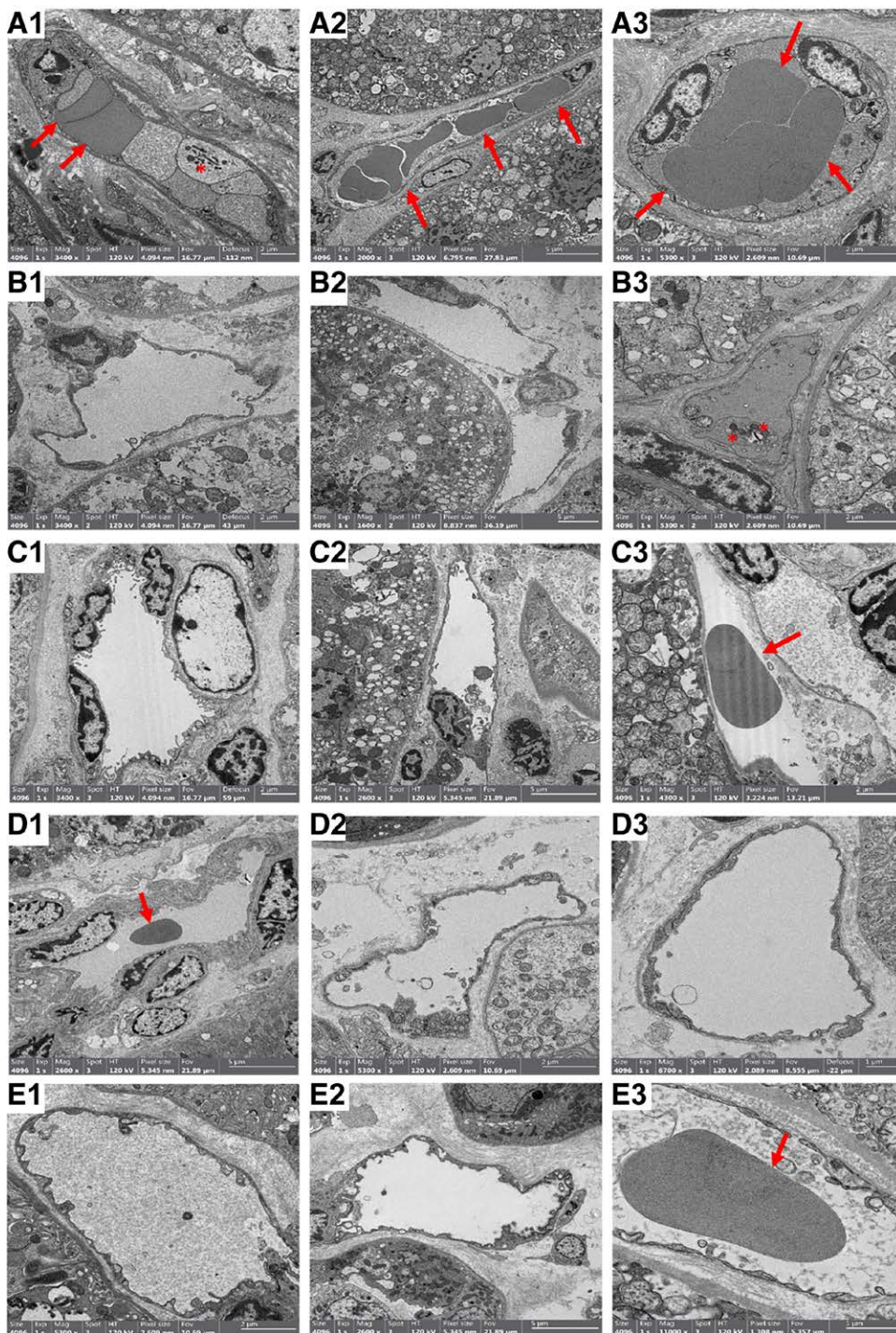
To evaluate kidney function at close to normal conditions and optimally buffer the system, the use of RBCs has been suggested to be superior to cell-free solutions,<sup>48</sup> although problems still remain to be solved.<sup>49</sup> The lung and heart ex vivo perfusion systems from the Steen group employ an oxygenated RBC-containing system.<sup>50</sup> This system has been used to recondition organs for many years, but not in the context of extended uDCD. We found that combining cold perfusion at low temperatures of 12–15 °C allows slow re-oxygenation and restoration of cell function and delivers excellent outcomes in the present study. The donor organ can be transported to the transplantation site during the cold perfusion phase, where it can be further reconditioned and evaluated using RBCs at a hematocrit of 10%–15%, which we found to be clinically relevant. Before transplantation, a decrease in temperature to 15 °C and a decrease in perfusion pressure to 20 mmHg was performed. Vascular resistance and arterial flow, in combination with the appearance of the organs, confirmed a functional kidney and indicated the range of renal flow expected at reperfusion after transplantation. Urine production was commonly observed, with signs of ability to concentrate urine, measured as increased creatinine in urine compared to the perfusion solution. Our

experience shows a preference for resistance levels under 200 Wood units, which is close to the values found in kidneys from live donors. Renal flow should also be higher than 100 mL/100 g tissue/min, which is consistent with a recent publication by Sandal et al.<sup>51</sup> Recipients with higher flows at reperfusion in our study also had a more favorable outcome (Figure 4), measured as the ability to survive beyond 5 d.

Histological analysis of the kidneys in the study group showed minimal changes, considering significant ischemic trauma. No significant differences or systematic changes between these groups, such as fibrosis, which could be attributed to the ischemia challenge, could be seen.

The functional outcome at 10 d and the actual number of recipients who succeeded after transplantation are encouraging and suggest that this will work in a clinical setting.

In summary, we suggest that uDCD organs, subjected to prolonged warm ischemia may be used as a source for organ transplantation, provided that fibrin clots in the capillaries are removed using a thrombolytic treatment during ex vivo perfusion and evaluation. Based on the protocol described in this article, we will undertake a preclinical kidney transplantation survival study over 3 mo, utilizing reconditioned kidneys from uDCD donors.



**FIGURE 9.** Representative images from biopsies before (A.1–A.3) and during machine perfusion (B.1–D.3) of uDCD kidneys (group I) with 4.5 h of WIT and of a normal kidney (E.1–E.3) using a high-resolution transmission electron microscopy. Fig A.1–A.3 shows fibrinogen and RBC rich microvascular plugs (red arrows) before any treatment in uDCD kidney biopsies 4.5 h after death. Platelets (marked in red asterisk) could be seen in many of the capillaries in biopsies taken before any treatment (A.1). No RBC plugs were found in biopsies taken after the flushing phase, although there were platelets observed in some of the capillaries (B.1–B.3). Capillaries appear clear and without any clots or platelets in biopsies taken at the end of the cold perfusion (C.1–C.3) and the end of the RBC phase (D.1–D.3). Capillaries in biopsies taken from normal pig kidneys (group II), can be seen as comparison in the last panel (E.1–E.3). RBCs are depicted as red arrows and platelets as red asterisks. RBC, red blood cell; uDCD, uncontrolled donation after circulatory deaths; WIT, warm ischemia time.

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