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ORIGINAL ARTICLE

One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation

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injury and preservation, injury mechanisms and biomarkers, ischemia/reperfusion injury, machine perfusion, organ preservation, pulsatile preservation.

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Summary

Studies have shown beneficial effects of machine perfusion (MP) on early kidney function and long-term graft survival. The aim of this study was to investigate whether the type of perfusion device could affect outcome of transplantation of deceased donor kidneys. A total of 50 kidneys retrieved from 25 donors were randomized to machine perfusion using a flow-driven (FD) device (RM3; Waters Medical Inc) or a pressure-driven (PD) device (LifePort; Organ Recovery Systems), 24 of these kidneys (n = 12 pairs; 48%) were procured from expanded criteria donors (ECD). The primary endpoints were kidney function after transplantation defined using the incidence of delayed graft function (DGF), the number of hemodialysis sessions required, graft function at 12 months, and analyses of biopsy. DGF was similar in both groups (32%; 8/25). Patients with DGF in the FD group required a mean of 4.66 hemodialysis sessions versus 2.65 in the PD group (P = 0.005). Overall, 1-year graft survival was 80% (20/25) vs. 96% (24/25) in the FD and PD groups. One-year graft survival of ECD kidneys was 66% (8/12) in the FD group versus 92% (11/12) in the PD group. Interstitial fibrosis and tubular atrophy were significantly more common in the FD group – 45% (5/11) vs. 0% (0/9) (P = 0.03) in PD group. There were no differences in creatinine levels between the groups. Machine perfusion using a pressure-driven device generating lower pulse stress is superior to a flow-driven device with higher pulse stress for preserving kidney function.

Introduction

Kidney transplantation has become the preferred method for treating patients with end-stage renal disease in current medical practice. In addition to immunologic factors, one of the obstacles to better early and long-term graft survival is ischemia reperfusion injury [1]. This might influence activation of innate immunity and development of interstitial fibrosis [2,3]. Several methods to minimize ischemia, such as pretreatment of donors, are difficult to introduce into clinical practice [4,5] or are still in the preclinical phase. A well-established method for tackling ischemic injury is the use of machine perfusion (MP) [6,7]. We have been using MP at our department for 18 years already. Published clinical evidence has demonstrated that MP reduces the incidence of delayed graft function (DGF) [7,8] and improves long-term graft survival [9,10] by minimizing the occurrence of interstitial fibrosis and tubular atrophy (IFTA) and chronic injury [11]. These benefits are most likely because of improved endothelial protection, influenced by activation of ischemic genes during reperfusion [12,13]. Machine

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perfusion is now being used for the preservation of other organs prior to transplantation, such as the liver, pancreas, heart or lungs [14–18].

Two of the most commonly used MP systems in clinical medicine are the pressure-driven (PD) device from Organ Recovery Systems, Chicago, IL, USA (LifePort®) and the flow-driven (FD) device from Waters Medical Systems, Rochester, UK (RM3). Both are used in combination with University of Wisconsin (UW) machine perfusion solution. In both systems, the operator can modify the systolic perfusion pressure, with flow and resistance indices recorded over time. Use of both these systems has been shown to be superior to cold storage [19]. A recent preclinical comparative study by Codas *et al.* [20] demonstrated minimal differences in early kidney recovery function between the two systems. However, significantly higher levels of fibrosis were observed 3 months postoperatively in kidneys preserved on the FD machine compared with the PD device [20]. To date, no clinical studies have compared flow-driven versus pressure-driven devices in kidney transplantation from deceased donors.

The aim of this study was to investigate whether the type of perfusion device used could have an impact on outcome in kidney transplantation from deceased donors.

Methods

Between August 2009 and January 2011, 50 kidneys retrieved from 25 deceased donors were enrolled in the study. A total of 24 kidneys (48%) were procured from 12 expanded criteria donors (ECDs). Kidneys were retrieved in hospitals outside our transplantation center and were transported on ice to our center. Immediately following organ recovery and cooling to 4 °C, each kidney was placed in a thermally stable container in a preservation solution (simple cold storage in UW solution).

Machine perfusion

On arrival at our transplant center, both kidneys were simultaneously prepared for machine perfusion. One kidney from each pair was placed on the flow-driven device (Waters RM3; Waters Instruments Inc.), and the contralateral kidney was placed on the pressure-driven device (LifePort; Organ Recovery Systems). Kidneys were kept perfused during preparation of the iliac fossa of the recipient before implantation. Kidneys were removed from the device just before vascular re-anastomosis in the recipient.

Randomization

A randomization schedule was applied to the left kidney, whereby alternate left kidneys were put on the FD or the PD device. To avoid a potential bias in respect of cold ischemia time (CIT) between the groups, the kidney placed on the PD device was alternately transplanted first or second. Pair of kidneys enrolled into the study could not differ between each other more than 15% in weight and had to have similar number of arteries (both one or both two or both more than two). Physicians who took care of patients after transplantation were blinded and did not have any knowledge about perfusion system used prior to transplantation. All biopsies were evaluated by "blinded" histopathologist who did not have any knowledge about perfusion system used prior to transplantation.

Flow-driven device (FD group)

After bench surgery, one kidney from each pair was placed on the flow-driven perfusion device within a sterile disposable cassette (MOX 100 DCM Disposable Cassette; Waters Instruments Inc.) filled with 1000 ml perfusion fluid (KPS-1; UW machine perfusion solution). A cooling bath was used to ensure a constant fluid temperature (4–6 °C) (Model 900 Constant Temperature Circulator; Fisher Scientific, Pittsburgh, PA, USA). A flow at a 1-Hz frequency (60 pulses/min) was set to achieve an initial systolic pressure of 45 mmHg. During MP (at 1, 2, 3 and 4 h and every 4 h thereafter), flow was manually adjusted according to the recommendations for use of the device (increase/decrease flow to keep systolic pressure at approximately 45 mmHg).

Pressure-driven device (PD group)

After bench surgery, the contralateral kidney from each pair was placed in a sterile disposable cassette filled with 1000 ml perfusion fluid (KPS-1, UW machine perfusion solution). Systolic pressure was set at 30 mmHg in accordance with the manufacturer's recommendations. During MP, flow was automatically adjusted by the pressure-driven processor of the device to maintain systolic pressure at 30 mmHg. The sterile disposable cassette was placed into a water–ice container to keep the temperature of perfusion between 4 and 6 °C.

Perfusion measurements

On both systems, the following parameters were monitored: systolic–diastolic pressure, mean perfusion pressure, flow per minute, and vascular resistance at 1, 2, 3, and 4 h and every 4 h thereafter during the perfusion period. Biochemical markers of ischemic organ injury were also investigated: lactic acid dehydrogenase (LDH; spectrophotometric assay) and lactate (GM7 APR) at the beginning of perfusion and at the fourth hour of perfusion.

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Biomarker analysis

The concentrations of caspase-3, tumor necrosis factor alpha (TNF-α), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1) in perfusion fluid were determined by enzyme-linked immunosorbent assay (ELISA). Protein concentrations were determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples of perfusion solution were taken at the beginning of perfusion (after 5–10 min) and then at the fourth hour of perfusion.

Study population

Fifty kidneys were transplanted into 50 recipients, 25 kidneys were perfused using the PD device and 25 using the FD device. Recipients of kidneys from the two perfusion systems did not differ in terms of HLA mismatch, age, gender, duration of dialysis treatment before transplantation and immunosupressive treatment after transplantation (Table 1). Tacrolimus dosage was level-dependent. In the first month in both groups, we kept levels at 12–15 ng/ml, then at 8–12 ng/ml and since the third month post-transplantation between 5 and 8 ng/ml. Ciclosporine dosage was also level-dependent. In the first 3 months post-transplantation, we kept C_2 level between 1000 and 1500 ng/ml, between 3 and 6 months post-transplantation we kept the level between 800 and 1000 ng/ml and after 6 months post-transplantation levels were maintained between 400 and 600 ng/ml.

Table 1. Recipients' characteristics (means ± SD).

	PD group (n = 25)	FD group (n = 25)	P-value
Age (years)	53.54 ± 13.6	46.4 ± 16	0.1
Male sex (%)	56	72	0.24
BMI (kg/m^2)	24.90 ± 4.64	24.65 ± 4.54	0.71
Duration of hemodialysis before transplantation (months)	39.86 ± 25.0	39.00 ± 41.0	0.91
HLA mismatch (A, B, DR)	4.77 ± 1.56	4.36 ± 1.65	0.81
Induction therapy	20% (5/25)	24% (6/24)	0.9
Triple drug immunosupression	100% (25/25)	100% (25/25)	1.0
Tacrolimus + Mycofenolane Mofetil + Steroids	80% (20/25)	68% (17/25)	0.52
Cyclosporine + Mycofenolane Mofetil + Steroids	20% (5/25)	32% (8/25)	0.52
Statin treatment at 1 year post-transplantation	68% (17/25)	56% (14/25)	0.56

Post-transplant analysis

Post-transplant analysis comprised several endpoints:

1. Immediate graft function – occurrence of DGF.
2. Numbers of hemodialysis (HD) sessions needed after transplantation.
3. Graft function 12 months after transplantation (serum creatinine level, graft survival, and albuminuria)
4. Analyses of biopsies.

Delayed graft function was recognized as a need for dialysis in the short term (7 days) after kidney transplantation, regardless of reason (hyperkalemia, high serum urea concentration).

IFTA and acute rejection were proven by biopsy and diagnosed according to Banff 2009 criteria. All kidneys had biopsies taken before transplantation as well as in case of deterioration of graft function later on. Patient and graft survival were analyzed in the 1-year post-transplant period. All patients completed the 1-year follow-up.

We considered 24-h proteinuria to be present if there was ≥200 mg protein in urine samples taken from 24-h urine collection at two consecutive examinations at least 6 months post-transplant.

Statistical analysis

Categorical variables in the two groups were compared with Chi-squared or Fisher's tests. Student's equivalent nonparametric t-test or Wilcoxon tests were used to test for differences between means and medians, respectively. Long-term outcome comparisons used Kaplan–Meier analysis with the log-rank test. Observation endpoints for the 1-year analysis were defined as either survival without hemodialysis [denoted as censored events (+)] or return to hemodialysis [complete (o)]. A critical α level for hypothesis testing was set at 0.05. Statistical version 9.0 software was used for the analysis.

Results

There were no differences in kidney weight before and after perfusion or in CIT between the two groups (Table 2). The mean CIT was 28 h ± 9 in the PD group versus 28 h ± 8 in the FD group (P = 0.68). Time of vascular anastomosis

Table 2. Perfusion parameters (means ± SD).

	PD group (n = 25)	FD group (n = 25)	P-value
Kidney weight before MP (g)	257 ± 67	274 ± 57	0.37
Kidney weight after MP (g)	319 ± 79	326 ± 70	0.79
Weight change (g)	67 ± 95	52 ± 25	0.93
Simple hypothermia before perfusion (minutes)	360 ± 100	360 ± 100	1
CIT total (hours)	28 ± 9	28 ± 8	0.68

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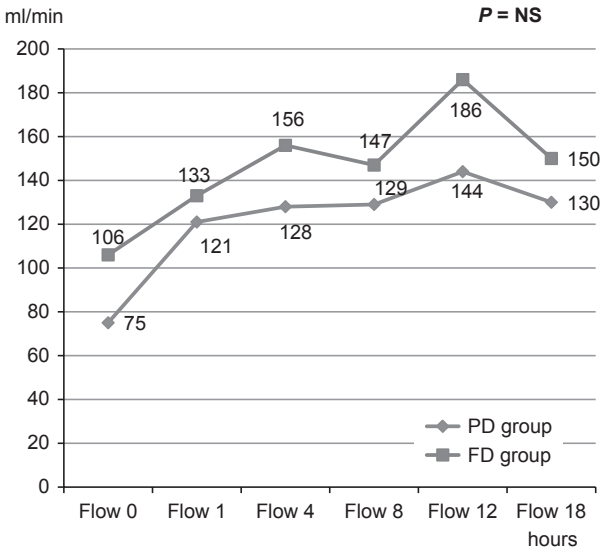

Figure 1 Renal flow during perfusion.

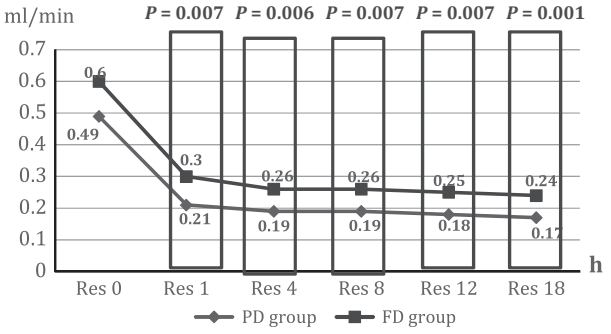

Figure 2 Renal resistance during perfusion.

was 31 min ± 9 in the PD group versus 29 min ± 11 in the FD group ($P = 0.78$).

Perfusion results

Renal flow was higher in the group of kidneys preserved with the FD machine, although the difference did not reach statistical significance (Fig. 1). Renal resistance, however, was significantly lower in kidneys preserved with the PD device from the first hour until the end of perfusion (Fig. 2). Mean perfusion pressure during the fourth hour of perfusion was significantly higher in the FD group than the PD group (31 vs. 22 mmHg, $P < 0.01$). Similar differences were found at the beginning and at the end of perfusion.

Biomarkers analysis (TNF-α, NGAL, KIM-1, and caspase-3)

There were no differences in biomarker activation in the perfusion solution at the beginning of perfusion. After the fourth hour of perfusion, mean activation of caspase-3 was statistically higher in perfusion samples taken from the FD compared with the PD group (Fig. 5). There were no differences in mean activation of TNF-α, NGAL or KIM-1 in perfusion solutions between the FD and PD groups at the fourth hour of perfusion (Table 3).

Table 3. Biomarker activation in perfusion solution.

	PD group	FD group	*P*-value
TNF-α – 0 h (ng/ml)	0.82	0.67	NS
TNF-α – 4th h (ng/ml)	0.96	0.67	NS
KIM-1 – 0 h (ng/ml)	0.068	0.138	NS
KIM-1 – 4th h (ng/ml)	0.254	1.359	NS
NGAL – 0 h (ng/ml)	4.81	7.48	NS
NGAL – 4th h (ng/ml)	11.08	12.02	NS

LDH and lactate

Mean LDH activation was significantly higher in samples of perfusion solution taken from the FD group compared with the PD group: 315.7 ± 126 U vs. 212.4 ± 77 U, respectively ($P = 0.004$). Mean concentration of lactate in perfusion solution at the fourth hour of perfusion did not differ between the groups (1.49 ± 0.70 mg/dl vs. 1.78 ± 0.73 mg/dl; $P = 0.2$).

Post-transplantation results

Delayed graft function did not differ between the groups, being 32% (8/25) in the PD group and 32% (8/25) in the FD group. Nevertheless, the mean number of hemodialysis sessions needed after transplantation in patients who had DGF was significantly lower in the PD group: 2.65 ± 1.5 vs. 4.66 ± 0.91 in the FD group ($P = 0.004$). Biopsy-proven episodes of acute rejection within 1-year of observation did not differ between the groups. The mean creatinine level did not differ statistically between the groups up to the end of 1-year of observation (Fig. 3).

One-year graft results

Overall, 1-year graft survival was 88% (44/50). One-year graft survival was 79% (19/24) for kidneys from ECDs and 96% (25/26) for kidneys retrieved from standard criteria donors. One-year graft survival was 96% (24/25) in the PD group versus 80% (20/25) in the FD group ($P = 0.07$) (Fig. 4). One-year graft survival of kidneys retrieved from ECDs was 92% (11/12) in the PD group versus 66% (8/12) in the FD group ($P = 0.09$).

Chronic proteinuria 1-year after transplantation was observed in 13% (3/24) in the PD group versus 25% (5/20) in the FD group ($P = 0.3$). Chronic proteinuria among the

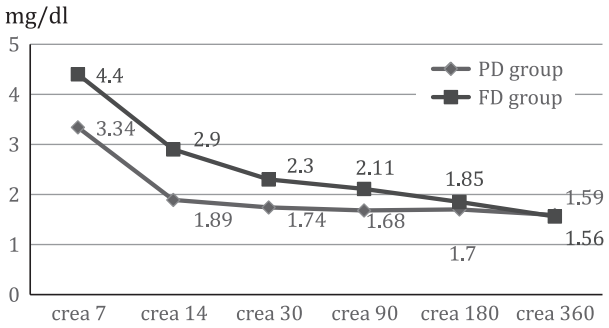

Figure 3 Mean serum creatinine level after kidney transplantation (P = NS).

patients who received kidneys from ECDs was observed in 9% (1/11) in the PD group versus 37% (3/8) in the FD group (P = 0.1).

Analysis of graft loss

One patient has lost graft function during observation period in PD group 4% (1/25) vs. 20% (5/25) in FD group (P = 0.06). Patient in PD group lost graft because of organ specific SSI. In FD group – one patient lost graft because of organ specific SSI, one because of organ failure as a complication of severe acute pancreatitis, one lost because of renal abscesses which developed several months post-transplantation, one because of sever lymphocele which eventually led to graft infection and graft function failure and fifth patient loss of graft function after ischemic heart failure – kidney biopsy revealed severe arteriosclerosis, glomerulosclerosis, and arteriole hyalinization (arteriosclerosis seen in biopsy taken prior to transplantation in kidney procured from ECD donor). Patient had constant poor function (best creatinine level 3.9 mg/dl – paired kidney from PD group also had arterioscelrosis in biopsy taken prior to transplantation but 1 year post-tx creatinine level in recipient was 1.9 mg/dl).

Analysis of biopsies taken within first year of observation

All kidneys had biopsies taken before transplantation. There were no differences in these biopsies. Acute tubular necrosis was seen in 80% (20/25) of biopsies from the PD group and 92% (23/25) of biopsies from the FD group. Arteriosclerosis was observed in 24% (6/25) PD group and 20% (5/25) FD group (P = 1.0). Glomerulosclerosis was spotted in none of PD group and in one case of FD group biopsies.

Within 1-year post-transplant, 20 patients had 22 biopsies for medical reasons (deterioration of graft function): nine patients from the PD group and 11 from the FD group. IFTA was present in five patients. All IFTA was mild in degree (<25% of fibrosis in cortical area). In the PD group, 0% (0/9) of patients had IFTA versus 45% (5/11) in the FD group (P = 0.01). T-cell mediated acute rejection was seen in 16% (4/25) in PD group versus 12% (3/25)

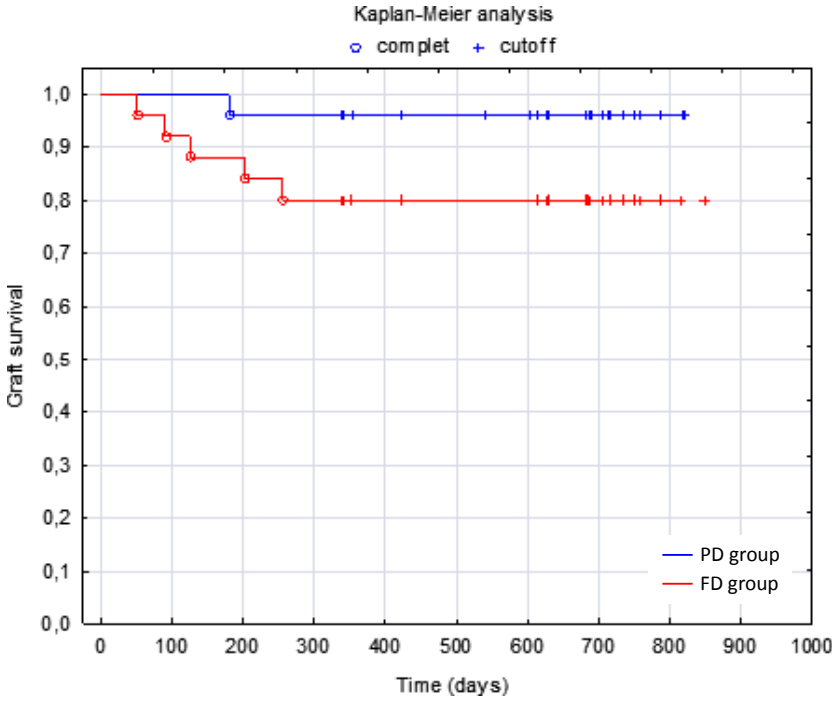

Figure 4 One-year graft survival (P = 0.07).

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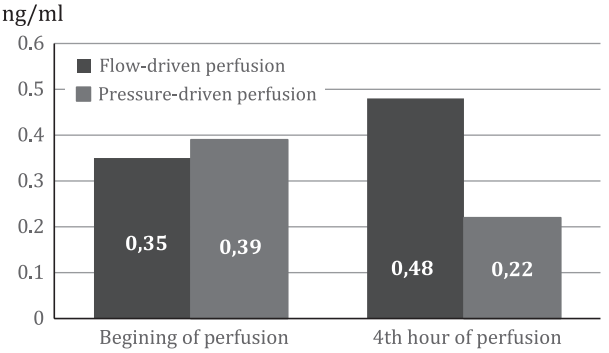

Figure 5 Mean caspase-3 activation in perfusion solution.

in FD group ($P = 1.0$). Borderline changes was seen in 4% (1/25) in PD group versus 8% in FD group ($P = 1.0$).

Results of patients diagnosed with IF/TA

Five patients from FD group were diagnosed with IF/TA within biopsies taken during first year post-transplantation. It did not affect graft survival – all graft had its function preserved after 1 year post-transplantation. Nevertheless, mean creatinine level was 2.2 ± 0.62 mg/dl in IF/TA patients versus 1.49 ± 0.42 mg/dl in the rest of analyzed population ($P < 0.05$).

Discussion

The aim of this study was to investigate whether the type of perfusion device could have an impact on outcome in kidney transplantation from deceased donors. To the best of our knowledge, this is the first prospective, randomized, controlled study comparing two renal perfusion devices being used in the clinic.

Both systems had already demonstrated superiority over cold storage in terms of postoperative kidney function and graft survival [9,10]. The beneficial effect of MP has been linked to improved protection of the endothelium [21]. Maintaining flow during preservation stimulates release of endothelial nitric oxide synthase (eNOS) leading to vaso-relaxation. Moreover, avoiding prolonged cessation of flow prevents loss of expression of endothelial protective genes such as Kruppel-like factor-2 [13]. Ischemic injury may lead to higher production of TGF-β and enhanced process of interstitial fibrosis [3]. Histopathologic analysis showed a significantly lower incidence of chronic rejection and interstitial fibrosis in kidneys preserved by MP [11].

Additionally, both systems use the same perfusate (KPS-1; UW-MP solution) and offer hypothermic perfusion to the kidney via the renal artery. However, there are some relevant differences between the systems in regards to the way these devices generate and control flow and pressure. Whereas the RM3 uses a flow-driven system, generating flow by squeezing a silicon tubing segment with unidirectional valves, LifePort uses a pressure-driven roller pump. Because of the different pump mechanisms, also different systolic pressures are being used. Given its specific pump mechanism; the flow-driven Waters device requires 50% higher systolic pressures (45 vs. 30 mmHg) to obtain similar flow rates. Using equal systolic pressures of 30 mmHg on the flow-driven device would have resulted in significantly lower flow rates during perfusion with unknown impact on preservation quality. The pressure settings which have been used during this study are in line with the recommendations of the manufacturer as well as with the extended experience with both devices at our center and other centers worldwide.

During our study, we registered significantly lower mean pressures on pressure-driven device. The use of lower perfusion pressures during hypothermic machine perfusion (HMP) has already been shown to result in lower activation of Von Willebrand factor (a marker of endothelial injury) [22], as well as in a better preservation of the kidney's structural integrity [23]. Maathuis *et al.* [22] reported less damage to the proximal tubuli, less reactive oxygen species, less pro-inflammatory cytokine release and better cortical perfusion when using perfusion pressures of 30/20 mmHg compared to 60/40. Also Doorschodt *et al.* [23] demonstrated better preservation of the structural integrity and improved recovery of renal function when using mean perfusion pressures of 25 mmHg compared to 30 mmHg.

Despite using 50% higher systolic pressures (45 vs. 30 mmHg) on FD device, we only observed slightly (non-significant) higher flow rates in this group. Kidneys preserved on the PD device opened up sooner and further, renal resistance in the PD group being significantly lower from the first hour until the end of the perfusion time. Although the lower renal resistance observed in the PD group might be partially because of the way in which renal resistance is calculated between both the systems (PD LifePort renal resistance = actual mean pressure/flow, FD RM3 renal resistance = systolic pressure + diastolic pressure + diastolic pressure/3/flow), this difference could not fully explain the significant lower renal resistance observed in the PD group. Nitric oxide release is responsible for vasodilatation and protects from hypertension [24–27] in humans. There is a hypothesis that in machine perfusion nitric oxide seems to acts similar [28]. Our observation fuels the hypothesis that nitric oxide release rather than the applied hydrostatic pressure is responsible for vasodilatation by machine preservation [28]. Based on the hypothesis of nitric oxide release being the basic mechanism behind vasodilatation of the renal microvas-

culature during perfusion, we hypothesize that lower nitric oxide release might possibly offer a plausible explanation for the higher renal resistances observed on the flow-driven device, but this needs further investigation.

With regard to the higher levels of LDH we recorded for the FD device, LDH is not only a general marker for hypoxia and cellular damage, but Herrera *et al.* [29] also found that higher levels of LDH were associated with higher renal pressures and kidneys showing more signs of interstitial and glomerular edema. These authors did not find any significant difference in LDH concentration for kidneys undergoing continuous versus pulsatile perfusion [29]. A possible explanation for the lack of a significant difference might have been that they analyzed perfusate samples taken at the second hour of perfusion, while in our study we analyzed perfusate samples at the fourth hour of perfusion. So, while searching for a plausible explanation for our observations, looking at the difference in perfusion pressures might certainly offer a valid option.

Higher concentrations of caspase-3 (a strong activator of apoptosis) in perfusate samples taken at the fourth hour of perfusion were also observed in kidneys preserved on the FD device, potentially reflecting a higher degree of injury. Caspase-3 inhibition with Si-RNA during the perfusion period allows for better creatinine clearance after transplantation in animal models and better blood flow in kidneys during the reperfusion period [30].

All previous observations point toward a more important level of injury in kidneys perfused with flow-driven devices with higher pressure. Again, using equal systolic pressures of 30 mmHg on the flow-driven device would have resulted in significantly lower flow rates during perfusion with, at that moment, unknown impact on preservation quality. Difference in flow with lower pressure might be explained by the difference in the creation of the pulse wave: The PD LifePort generates a sinusoidal pulsatile waveform, whereas the FD RM3 creates a more physiologic pulse wave. The team from Newcastle [31] invalidated the presumed need for and benefit of pulsatility during HMP in kidneys. The latter group demonstrated even slightly better eGFR, graft and patient survival up to 5 years post-transplant in DCD kidneys perfused by continuous MP compared with those perfused by pulsatile perfusion. These results might be explained by the fact that flow pattern is laminar during continuous perfusion.

In our study, in post-transplant period, we observed exactly the same level of incidences of DGF in both groups but DGF in FD group lasted significantly longer. During 1-year observation period, incidences of chronic proteinuria were more often (although not statistically significant) observed in the FD group. Significantly higher incidences of IF/TA were observed also in FD group. Those results confirm the findings from the pre-clinical study by Codas *et al.* [20], which found higher incidences of interstitial fibrosis in kidneys that had been preserved on flow-driven device. As there were no differences in donors, transplant procedure and recipients, one might expect that difference should be, somehow, connected with preservation and the difference in devices which were used. Under physiologic conditions, arterial dampening of the pressure oscillations limits their transmission to the microcirculation [32]. Nephrologists recognize arterial hypertension as a risk factor in patients with atherosclerosis; it may lead to proteinuria and eventually loss of kidney function. Maintaining autoregulation has been identified as an important protective mechanism in highly perfused organs with low arteriolar resistance such as the kidney. However, if vascular compliance is reduced by increased rigidity of successive arteries (for example, hypertension or hypothermia), these firmer pulses may not be dampened sufficiently. Therefore, these pulses are more likely to reach and damage the microcirculation of the kidney. Moreover, endothelial injury might trigger endothelial-to-mesenchymal transformation of pericytes [3], resulting in myofibroblast production with higher collagen IV production and interstitial fibrosis. Although, it was never the aim of our study to identify or validate any potential mechanisms which could help to explain our observations, recent publications seem to indicate that applying gentle pressures and pulse waves during HMP might be beneficial to limit the risk of damaging the glomeruli [32]. In the setting of HMP, hypothermia reduces vascular compliance; making the ischemic, fragile endothelium even more vulnerable to pulse stress. These risks are indirectly confirmed by our observations of higher renal resistance and higher levels of LDH and caspase-3, as well as by higher incidences of chronic proteinuria and IFTA with the FD device. Because of small study groups, power of those results should be evaluated again and should encourage for definitely larger study.

Conclusion

In our study, perfusion of kidneys with the PD device prior to transplantation compared with perfusion using the FD device resulted in:

1. Shorter duration of DGF after kidney transplantation.
2. Lower incidences of IFTA in biopsies taken within 1-year post-transplantation.
3. Lower renal resistance during perfusion.

Authorship

WM: designed research/study, performed research/study, collected data, analyzed data, and wrote the paper. KA: designed research/study, and contributed important reagents. DP and GL: performed research/study, and collected data. DP: designed and performed research/study.

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KR and BA: collected data. PA, DM and PL: contributed important reagents. CA: designed research/study.

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