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ORIGINAL ARTICLE

First clinical-grade porcine kidney xenotransplant using a human decedent model

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A radical solution is needed for the organ supply crisis, and the domestic pig is a promising organ source. In preparation for a clinical trial of xenotransplantation, we developed an *in vivo* pre-clinical human model to test safety and feasibility tenets established in animal models. After performance of a novel, prospective compatible crossmatch, we performed bilateral native nephrectomies in a human brain-dead decedent and subsequently transplanted two kidneys from a pig genetically engineered for human xenotransplantation. The decedent was hemodynamically stable through reperfusion, and vascular integrity was maintained despite the exposure of the xenografts to human blood pressure. No hyperacute rejection was observed, and the kidneys remained viable until termination 74 h later. No chimerism or transmission of porcine retroviruses was detected. Longitudinal biopsies revealed thrombotic microangiopathy that did not progress in severity, without evidence of cellular rejection or deposition of antibody or complement proteins. Although the xenografts produced variable amounts of urine, creatinine clearance did not recover. Whether renal recovery was impacted by the milieu of brain death and/or microvascular injury remains unknown. In summary, our study suggests that major barriers to human xenotransplantation have been surmounted and identifies where new knowledge is needed to optimize xenotransplantation outcomes in humans.

KEYWORDS
clinical research/practice, genetics, kidney transplantation/nephrology, translational research/science, xenoantigen, xenotransplantation

Abbreviations: 10-GE pigs, 10 gene edited pigs; CNI, calcineurin inhibitor; DAF, decay accelerating factor; DRC, donor recovery center; HLA, human leukocyte antigen; NHP, non-human primate; PERV, porcine endogenous retrovirus; SLA, swine leukocyte antigen; TBM, thrombomodulin; XPC, xenotransplantation procurement campus.

1 | INTRODUCTION

For most of the more than 700 000 Americans living with kidney failure,[1] kidney transplantation—the gold standard treatment—remains elusive,[2] despite efforts to increase the donor pool.[3-5] The domestic pig is a promising source of kidney xenografts. Proof-of-concept work has been performed in pig to non-human primate (NHP) models, in which NHPs were thought to best recapitulate human biology.[6] Critically, this work identified a major immune barrier to xenotransplantation—namely, the existence of carbohydrate antigens on vascular endothelium that are not expressed in Old World NHPs and humans. Genetic modifications to remove these antigens have improved the outcome of porcine xenotransplants in NHPs by avoiding hyperacute rejection.[7-9] Additional modifications designed to mitigate complement-mediated cytotoxicity and thrombosis have further refined the model.[10,11]

Despite the power of the NHP model, it is unlikely that all immunologic and functional hurdles will be overcome given the many biologic differences that exist between NHPs and humans. For example, NHP models are inadequate to prospectively test crossmatching assays for human use. Moreover, there are safety concerns surrounding transmission of porcine viruses,[11-13] and whether porcine genetic modifications are sufficient to avert hyperacute rejection in humans can only be determined through *in vivo* human studies. Ultimately, a human xenotransplantation experience will be required to develop the necessary knowledge to achieve excellent outcomes in humans. Given the inevitability of this bold step into human testing, the primary questions confronting the field are thus *when* and *how* to make this leap. In light of the extreme lethality of the organ shortage crisis and the successes achieved thus far in NHP models, one could argue that human testing is perhaps overdue. Nevertheless, caution is warranted, and some degree of efficacy must be expected. One-off experiments performed outside of a committed xenotransplantation program that do not address key knowledge gaps should be discouraged.

Consequently, the xenotransplantation program at the University of Alabama at Birmingham was started in 2015. The overarching goal of the program is to advance xenotransplantation into the clinical realm in a robust, sustainable, and ethical manner. Requisite high-level investments have included (1) the building of a designated pathogen-free animal facility in proximity to the transplant center committed only to human xenotransplantation, (2) the recruitment and training of a multidisciplinary team with extensive experience in human translational research as well as incompatible kidney transplantation, and (3) the establishment of key partnerships with industry and regulatory agencies that are necessary to move xenotransplantation into human recipients. As part of a stepwise approach into human xenotransplantation testing, we sought to develop a human pre-clinical model that would address fundamental questions regarding the safety and feasibility of porcine xenotransplantation into humans. This approach is founded on the premise that such questions must be answered before clinical trials of efficacy can be responsibly undertaken. We hypothesized that human brain death might provide the necessary model to examine safety and feasibility.[14] Although brain death pathophysiology may create a hostile environment for transplantation and limit assessment of kidney function,[15] such a model would allow for *a priori* assessment of multiple risks, including hyperacute rejection through the use of a xenotransplant-specific prospective crossmatch, life-threatening surgical complications, and viral transmission, thereby facilitating the development of the first phase I clinical trial in living humans. Herein, we present the results of the first clinical-grade xenotransplant experience *in vivo* using a human decedent model.

2 | MATERIALS AND METHODS

2.1 | Oversight and study location

1. Porcine kidney procurement was performed in an external surgical suite adjacent to a pig facility free of designated pathogens on the Xenotransplantation Procurement Campus (XPC) of the University of Alabama at Birmingham Heersink School of Medicine (UAB). Oversight of all activities at the XPC is provided by the Institutional Animal Care and Use Committee (IACUC-22015).
2. Xenotransplantation of the decedent was performed at the Legacy of Hope Donor Recovery Center (DRC) with institutional approval from UAB (IRB-300004648).

Additional facility details are included in Supplementary Information.

2.2 | Decedent inclusion and exclusion criteria

Eligible human decedents included adults (≥18 years), declared brain-dead, referred for organ donation but ruled out for donation of heart, lung, liver, pancreas, and/or intestine, whose next-of-kin authorized research and transport to the recovery center, and had a negative prospective crossmatch with the donor pig.

2.3 | Source animals

Porcine renal xenografts were procured from genetically engineered (GE) pigs provided by Revivicor, Inc. The GE pigs harbor ten genetic modifications (10-GE pigs), including targeted insertion of two human complement inhibitor genes (hDAF, hCD46), two human anticoagulant genes (hTBM, hEPCR), and two immunomodulatory genes (hCD47, hHO1), as well as deletion (knockout) of 3 pig carbohydrate antigens and the pig growth hormone receptor gene. Importantly, 10-GE pigs do not express red blood cell antigens and are therefore universal donors with respect to blood type.

2.3.1 | Development of 10-GE pigs

Two multi-cistronic vectors were generated that contained 2 human genes (*DAF* and *CD46*) or 4 human genes (*TBM*, *EPCR*, *CD47*, *HO1*), where the genes were separated by 2A sequences to achieve co-expression once introduced in pig cells. Correct, single copy targeting of these transgene constructs to landing pads was confirmed by PCR, Southern blot, and digital drop PCR. Knockout (KO) of α-1,3-galactosyltransferase (GGTA1, the enzyme responsible for synthesis of Gal) was confirmed by PCR for the presence of a disruptive insertion in exon 9. KO of genes encoding β1,4-N-acetylgalactosyltransferase (β4GalNT2, the enzyme responsible for synthesis of SDa), CMP-N-acetylneuraminic acid hydroxylase (CMAH, the enzyme responsible for synthesis of Neu5Gc) and growth hormone receptor (GHR) were assessed by Next-Gen DNA sequencing (MiSeq, Illumina) for the presence of large or frameshifting indels. Phenotypes of GGTA1KO, B4GALNT2KO, and CMAHKO were confirmed by flow cytometry of PBMC stained with IB4 lectin, DBA lectin and anti-Neu5Gc respectively, to reveal the absence of xenogeneic carbohydrate residues catalyzed by the knocked-out gene product. GHRKO phenotype was determined by demonstrating reduced serum IGF-1 levels and body weight. Expression of individual transgenes was confirmed in kidney biopsies of the donor pig after transplantation by western blot and immunohistochemistry.

2.3.2 | Housing and maintenance of 10-GE pigs

The 10-GE pigs are housed in facilities on the UAB XPC and are free of specified infectious agents (e.g., porcine CMV and porcine endogenous retrovirus C) which is assured by rigorous documentation, maintenance of well-defined routine testing, and rigorous standard operating procedures and practices for herd husbandry and veterinary care. Donor source 10-GE pigs are tested every three months for porcine viruses, including porcine endogenous retrovirus C (see Table 4). All testing is performed at the University of Minnesota Veterinary Diagnostic Laboratory (https://www.vdl.umn.edu/).

2.4 | Histocompatibility testing

Serologic compatibility was assessed between the donor pig and human decedent (recipient) prior to transplant. 10-GE donor lymphocytes were targets in a flow cytometric crossmatch with pre-xenotransplant decedent serum. Negative control was pooled human male AB serum. Positive control serum was human serum containing IgG known to react with porcine cells. For all tubes, 400 000 cells and 40 μl serum were incubated with FITC-conjugated goat anti-human IgG F(ab)'2 (Jackson ImmunoResearch Laboratories). Acquisition and analysis of flow crossmatch results were performed on a Beckman Coulter Cytoflex Flow Cytometer. Median channel shift of decedent sample was compared to negative and positive control sera to determine positivity.

2.5 | Viral and chimerism testing

RNA was isolated from human PBMCs and pig tissues, following standard protocols (Direct-zol RNA Miniprep, ZymoResearch). For cDNA synthesis, 50–100 ng of DNAse treated mRNA was reverse transcribed using an oligo-dT primer and the GoScript Reverse Transcription System (Promega). For the PCR reaction, 1 μl of cDNA template or water and 0.2 μM of each primer were added to 1x EmeraldAmp GT PCR Master Mix (Takara Bio) and amplified for 35 cycles of denaturation (98°C/1 min), annealing (60°C/30 s), and extension (72°C/45 s). The RT-PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide and visualized (FluorChem R imager, ProteinSimple).

2.6 | Surgical procedures

2.6.1 | Kidney procurement

After induction of general anesthesia, the 10-GE pig donor kidneys were procured *en bloc* in a standard operating room at the UAB XPC using an aseptic technique.

2.6.2 | Decedent bilateral native nephrectomies

In an operating room meeting The Joint Commission standards, bilateral native nephrectomies were performed using a standard open donor nephrectomy technique to establish anuria and to allow the kidneys to be used for allotransplantation.

2.6.3 | Backbench preparation of the porcine kidney xenograft

En bloc kidneys were separated, and pre-implantation biopsies were obtained. While grossly normal, the porcine kidneys and the accompanying vascular structures were soft on palpation with an extremely thin capsule and reduced gross structural integrity compared to human kidneys. In addition, the ureters were larger in diameter than typically observed in human kidneys. These observations underscored the need for meticulous handling and surgical technique.

2.6.4 | Porcine kidney xenotransplantation

Right and left 10-GE pig kidneys were transplanted separately using conventional heterotopic allotransplantation techniques. The right ureter was anastomosed to the decedent's bladder, and the left ureter was brought through the skin as an end urostomy. A post-reperfusion biopsy of the left porcine renal xenograft was obtained in vivo. Due to the delicate nature of the porcine tissues,

a complementary biopsy of the right porcine xenograft at this time point was deferred.

2.7 | Immunosuppression

Induction immunosuppression consisted of daily methylprednisolone taper, anti-thymocyte globulin for a total of 6 mg/kg, and anti-CD20. Maintenance immunosuppression included mycophenolate mofetil, tacrolimus, and prednisone.

2.8 | Histology

Biopsies were formalin fixed and sectioned for staining including PASH, immunohistochemistry, hematoxylin & eosin, silver, and immunofluorescence in standardized methods. Formalin fixation was performed in order to reduce potential infectious risk.

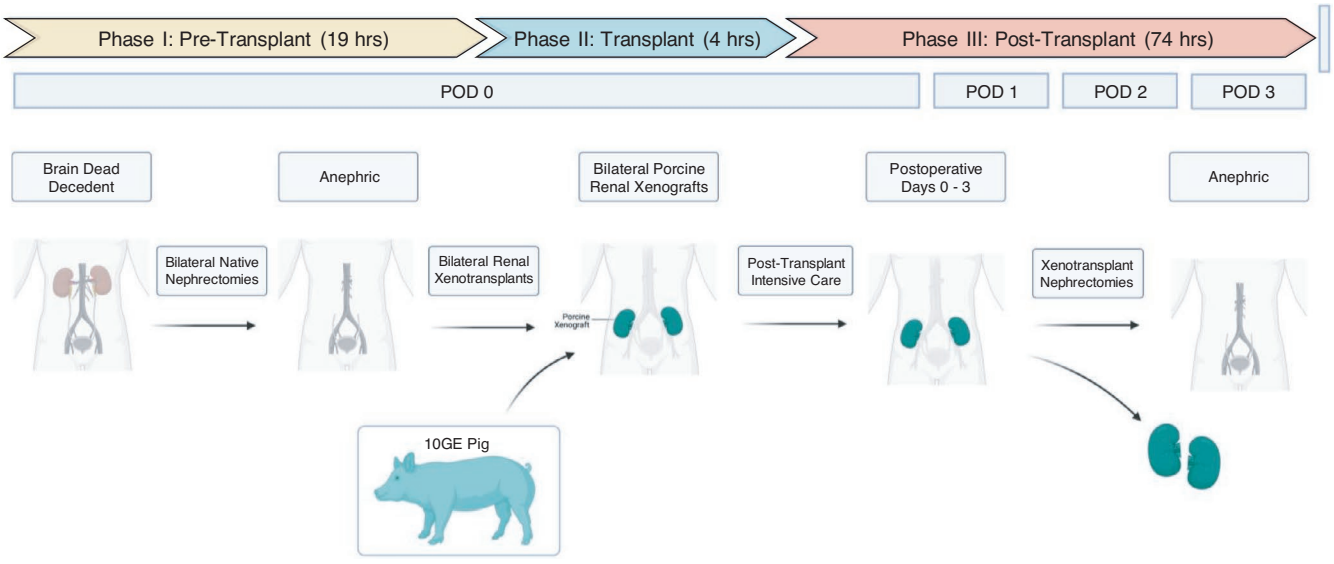
2.9 | Data management

Data were input in real time in a secure REDCap database by study personnel. The decedent was given an alias to preserve anonymity during the course of the study. All study personnel were aware of and instructed on the need to maintain the strictest of confidence about this study. All study personnel have received requisite training in data confidentiality and human subjects research.

3 | RESULTS

3.1 | Study overview and outcome measures

To test the core principles of the pig-to-NHP model in humans without risk to a living human being, we designed a safety and feasibility study of kidney xenotransplantation using a human brain-dead decedent model that included a pretransplant phase (19 h), a transplant phase (4 h), and a posttransplant phase (74 h) (Figure 1). The primary goal of the study was to address core safety questions within the limits of the decedent model that would inform the development of an IRB-approved clinical trial (Table 1). A secondary goal was to test our xenotransplantation program infrastructure by executing all the steps required to perform kidney xenotransplantation in living humans. Of note, efficacy measures were collected as tertiary outcomes as we did not expect the altered physiologic milieu of brain death to provide an optimal environment to support renal recovery. Nevertheless, we wanted to capitalize on the opportunity to collect



• Family authorization • Prospective crossmatch • Xenograft procurement • Pre-transplant xenograft histology • Removal of bilateral decedent native kidneys	• Induction immunosuppression • Transplantation of right and left kidney xenografts • Visual assessment for hyperacute rejection • Visual assessment of xenograft perfusion • Post-implantation xenograft biopsies	• Induction and maintenance immunosuppression • Assessment of xenograft function • Visual inspection of xenografts, including vascular and ureteral anastomoses • Assessment of xenograft perfusion • Assessment of sensitization (anti-HLA antibodies) • Assessment for transmission of porcine endogenous retroviruses • Serial xenograft biopsies • Bilateral xenograft nephrectomies

FIGURE 1 Study timeline and event summary. Created with BioRender.com

TABLE 1 Summary of study goals

Question	Phase	Metric
Genetic engineering sufficient to prevent hyperacute rejection?	Intraop	Gross appearance of kidneys
Negative prospective crossmatch sufficient to prevent hyperacute rejection?	Intraop	Gross appearance of kidneys
Life-threatening intraoperative complication?	Intraop	1. Vascular integrity 2. Hemodynamic stability during reperfusion
Porcine-derived products detectable in human blood?	Postop	1. PERV-C transmission 2. Presence of porcine proteins ubiquitous to all cells (i.e., ribosomal components)
Execution of best practices as needed for future xenotransplantation clinical trial?	All phases	Varies with regulatory agency

functional data as allowed within the constraints of the model; decedent bilateral native nephrectomies were therefore performed prior to xenotransplantation to permit interpretation of serum creatinine and other parameters of renal function (Figure 1).

3.2 | Pretransplant phase

After exhausting the solid organ transplant lists, next-of-kin was approached regarding decedent study enrollment and provided informed consent authorizing the participation of the 57-year-old brain-dead male (Table 2). At the time of enrollment, the decedent was 5 days post-declaration of brain death and had mild-to-moderate acute kidney injury (Table 3). The decedent was maintained on phenylephrine (1 mcg/kg/min), vasopressin (0.008 units/min), levothyroxine (10 mcg/h), and methylprednisolone with normal hemodynamics (BP: 178/92, HR: 61, temp: 98.8) as per routine management of brain-dead individuals prior to organ donation. A 13-month-old, 350 lb, male 10-GE donor pig (Figure S1) was identified at the UAB Xenotransplantation Procurement Campus (XPC). The donor animal had normal renal function (BUN 19, creatinine 1.3, assessed <60 days prior to donation) and was negative for porcine endogenous retrovirus C and other pathogens (Table 4). Prospective flow crossmatch between the decedent and 10-GE pig was negative (Figure 2).

The decedent was brought to an operating suite in the UAB Donor Recovery Center (DRC), and anuria was established by performing bilateral native nephrectomies (Figure S2). Simultaneously, surgical procurement of the porcine kidneys occurred in an operating suite at the XPC (Figure S3). Of note, a surgical injury to the left porcine renal vein during procurement was repaired intraoperatively after clamping of the left renal vein for approximately 20 min. The kidneys were packaged in sterile fashion and transported on ice from the XPC to the DRC. Backtable preparation of the porcine kidneys occurred in standard fashion (Figure S4A). Anatomy of the porcine kidneys largely recapitulated human renal anatomy. Pre-implantation biopsies demonstrated normal histology of the 10-GE pig kidneys that appeared similar to normal human kidney (Figure S4B).

3.3 | Transplant phase

The 10-GE pig kidneys were transplanted sequentially into the decedent using conventional heterotopic allotransplantation technique. Of note, the kidneys were transplanted into the bilateral iliac fossae, thereby replicating the retroperitoneal location used in most kidney transplant centers. Warm ischemia time was 28 and 29 min for the right and left xenografts, respectively; cold ischemia time was 4 h and 5 h 37 min for the right and left xenografts, respectively. Although results from some NHP studies suggest that calcineurin inhibitor (CNI)-based immunosuppression regimens may not be as effective as CD40-based regimens,[16] the precise mechanisms underlying graft loss in these NHP experiments are unknown and may not apply to human immune populations. As CNIs are highly effective in the prevention of cellular rejection in humans and the backbone of virtually all immunosuppression regimens in contemporary allotransplantation, we selected a conventional immunosuppression regimen to mimic "real-world" conditions of xenotransplantation. Methylprednisolone and anti-thymocyte globulin were thus administered immediately prior to xenotransplantation, and tacrolimus-based maintenance immunosuppression was started and maintained throughout the remainder of the study with effective depletion of lymphocytes (Table 5, Figure S5).

Both kidneys reperfused promptly with excellent color and turgor as judged independently by four experienced kidney transplant surgeons (Figure 3). Pulses were confirmed in the renal arteries with direct visual inspection and manual palpation. Doppler signals were normal in both the kidney parenchyma and the renal arteries bilaterally. There was no significant bleeding of the anastomotic suture lines or disruption of the renal parenchyma despite perfusion of the kidney with a human mean arterial blood pressure. The decedent remained on relatively stable doses of phenylephrine and dopamine prior to and after reperfusion (Figure 4). The right kidney made urine within 23 min of reperfusion. Urine output from the left kidney was more sluggish. The kidneys were observed under direct vision for at least 60 min prior to commencement of the ureteral anastomoses. No hyperacute rejection was observed and both kidneys maintained good color and turgor throughout the remainder of the operation. Post-reperfusion biopsy of the left kidney demonstrated mild to

TABLE 2 Decedent demographics and pertinent history

Characteristic	Decedent
Sex	Male
Race	White
Age	57 years
BMI	35.2 kg/m^2
Cause of death	Head trauma
Mechanism of injury	Blunt trauma
Past medical history	Hypertension, Hyperlipidemia
Past surgical history	Trauma exploratory laparotomy
Blood type	AB+
Calculated panel reactive antibody (cPRA)	0%

moderate acute tubular injury and normal glomeruli. There was no evidence of endothelial injury, fibrin thrombi, or staining for IgG, IgM, or C4d (Figure 3).

3.4 | Posttransplant phase

The decedent was maintained in the operating room for the remainder of the study. He received intensive nursing care, monitoring, and laboratory investigations as required for maintenance of cardiovascular perfusion in the setting of brain death. Over the ensuing three days of the study, the decedent developed progressive multisystem organ failure with evidence of shock liver, pancytopenia, and disseminated intravascular coagulation (Figures S6–S8). Acidemia was significant, and maintenance of a normal pH and serum bicarbonate level (Figure S9) required continuous administration of sodium bicarbonate (i.e., sodium bicarbonate 150 mEq + Dextrose 5% in Water @ 50 ml/h daily). He received continuous infusion heparin, blood transfusions, and additional high dose methylprednisolone to counter the effects of brain death physiology. Despite the severity of his physiologic derangement, his hemodynamics were sufficiently maintained to permit longitudinal data collection and exploration of the abdomen on days 1 and 3 for biopsies and kidney visualization (Figure S10). Notably, the kidney xenografts were well-perfused with the maintenance of turgor and Doppler signals throughout the parenchyma at all time points (Figure 5). Two hours after the surgical exploration on day 3, the decedent developed exsanguinating hemorrhage due to his severe coagulopathy. The study was thus terminated at 77 h and 32 min after reperfusion and 8 days post-declaration of brain death.

The right kidney made 700 cc of urine within the first 24 h, with scant urine production from the left (Figure 6). Urine output from each kidney was monitored separately as the right xenograft ureter was anastomosed to the decedent's bladder while the left xenograft ureter was exteriorized as a urostomy. Urinalysis obtained from the right kidney on post-operative day 1 (POD 1) revealed a normal specific gravity and the presence of RBCs, mild proteinuria and mild glucosuria (Table 6). Serum creatinine did not decrease over the course of the study (Figure 6), and neither kidney excreted significant creatinine into the urine (Table 7, results shown for right kidney). However,

TABLE 3 Decedent baseline laboratory results upon arrival to the Legacy of Hope donor recovery center

Analyte	Decedent	Normal reference range
Sodium (mMol/L)	**153**	133–145
Potassium (mMol/L)	4.6	3.1–5.1
Chloride (mMol/L)	**121**	97–108
Bicarbonate (mMol/L)	23	22–32
Anion gap (mMol/L)	9.0	4.0–16.0
Glucose (mg/dL)	**163**	70–100
BUN (mg/dL)	**48**	5–22
Creatinine (mg/dL)	**2.5**	0.7–1.3
Magnesium (mg/dL)	2.6	1.7–2.5
Calcium (mg/dL)	**7.7**	8.4–10.4
Protein (gm/dL)	**4.8**	6.0–8.3
Albumin (gm/dL)	**2.7**	3.7–5.5
Phosphorus (mg/dL)	4.3	2.3–4.6
Total bilirubin (mg/dL)	**1.5**	0.3–1.4
Direct bilirubin (mg/dL)	**0.7**	0.0–0.3
Indirect bilirubin (mg/dL)	0.8	0.3–1.0
Alkaline phosphatase (units/L)	116	37–117
ALT (units/L)	**411**	7–52
AST (units/L)	**90**	12–39
WBC (10^3/cmm)	8.21	4.00–11.00
RBC (10^6/cmm)	**3.21**	4.40–5.80
Hemoglobin (gm/dL)	**10.1**	13.5–17.0
Hematocrit (%)	**29**	39–50
Platelet (10^3/cmm)	**61.1**	150.0–400.0
Neutrophils (%)	**88**	35–73
Abs. neutrophils (10^3/cmm)	7.22	1.82–7.42
Lymphocytes (%)	**3**	15–52
Abs. lymphocytes (10^3/cmm)	**0.23**	1.25–5.77
Monocytes (%)	9	4–13
PT (seconds)	**15.6**	12.0–14.5
INR	**1.23**	0.9–1.1
PTT (seconds)	29	25–35
D-dimer (ng/mL DDU)	**>20 000**	0–240
pH	7.36	7.35–7.45
PCO_2 (mmHg)	40.0	35.0–45.0
PO_2 (mmHg)	93	80–100
HCO_3 (mMol/L)	22.8	22.0–26.0

Note: Abnormal results are depicted in bold.

TABLE 4 Results of pathogen screening of the donor pig

Test	Methodology	Sample	Result	Date
Hepatitis E	Real-time PCR	Feces	Negative	September 7, 2021
Herpes virus gamma	PCR	Buffy coat	Negative	September 7, 2021
Influenza A	Real-time PCR	Nasal Swab	Negative	August 13, 2021
Mycoplasm hyopneumoniae	Real-time PCR	Nasal Swab	Negative	August 16, 2021
Porcine circovirus 2,3 (duplex)	Real-time PCR	Serum	Negative	August 18, 2021
Porcine cytomegalovirus	Real-time PCR	Buffy coat	Negative	August 17, 2021
Porcine endogenous retrovirus A	PCR	Buffy coat	Positive 20.23 Ct40	August 19, 2021
Porcine endogenous retrovirus B	PCR	Buffy coat	Positive 21.65 Ct40	August 19, 2021
Porcine endogenous retrovirus C	PCR	Buffy coat	Negative	August 19, 2021
Porcine Epidemic Diarrhea Virus (S gene)	Real-time PCR	Feces	Negative	August 13, 2021
Porcine deltacoronavirus	Real-time PCR	Feces	Negative	August 13, 2021
Transmissible Gastroenteritis virus	Real-time PCR	Feces	Negative	August 13, 2021
Porcine reproductive and respiratory syndrome virus (PRRSV) European	Thermo Fisher Real-time PCR	Serum	Negative	August 13, 2021
Porcine reproductive and respiratory syndrome virus (PRRSV) North American	Thermo Fisher Real-time PCR	Serum	Negative	August 13, 2021

Note: Most recent testing results in advance of the procurement are shown. Results depicted below reflect testing for pathogens that impact porcine and/or human health and do not all have zoonotic potential. All testing was performed at the University of Minnesota Veterinary Diagnostic Laboratory. Test dates reflect completion of the assay.

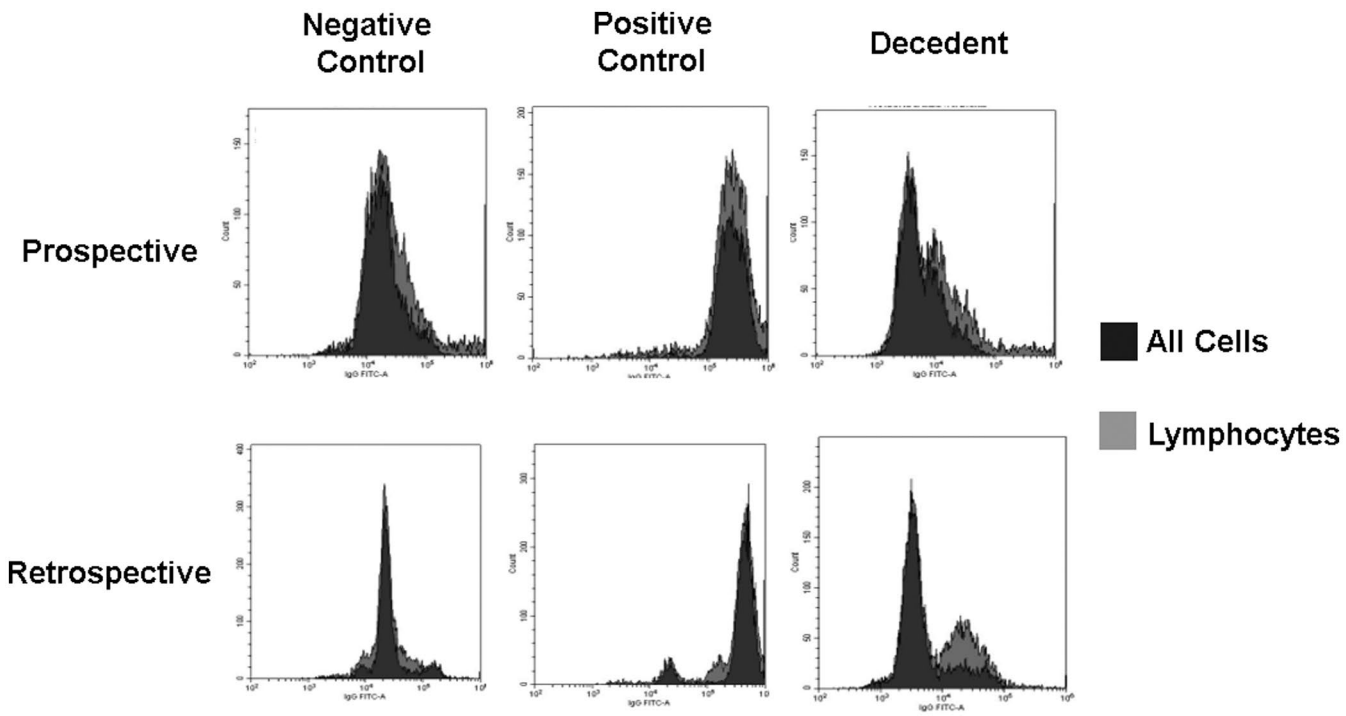


FIGURE 2 Detection of swine leucocyte antigen and decedent flow crossmatch results. PBMCs from a 10GE pig were isolated and incubated with pretransplant sera from the decedent. Porcine PBMCs were also incubated with negative and positive control sera that were identified from screening of sera banked in the histocompatibility laboratory at the University of Alabama at Birmingham. Anti-FITC secondary antibody (goat) was used to detect antibodies in the serum that were bound to the porcine lymphocytes. Histograms are shown for all cells or for lymphocytes gated based on FSC and SSC characteristics. Prospective crossmatches were performed using frozen porcine PBMCs. Retrospective crossmatches were performed using freshly isolated porcine PBMCs

TABLE 5 Pharmacologic immunosuppression regimen

Immunosuppressive medication	POD 0	POD 1	POD 2	POD 3
Anti-Thymocyte Globulin (Rabbit)	175 mg	175 mg	175 mg	—
Rituximab	1800 mg	—	—	—
Tacrolimus	— 1 mg PM	1 mg AM 1 mg PM	1 mg AM 2 mg PM	2 mg AM —
Mycophenolate mofetil	— 2000 mg PM	1000 mg AM 1000 mg PM	1000 mg AM 1000 mg PM	1000 mg AM —
Methylprednisolone[a]	500 mg	250 mg	125 mg	90 mg

Abbreviations: POD AM, morning of post-operative day; POD PM, afternoon of post-operative day.

[a] Additional methylprednisolone given for brain death management.

normal serum electrolytes were maintained (Figure S9), likely due in part to exogenous administration of sodium bicarbonate.

Histologic findings on post-operative day 1 were consistent with thrombotic microangiopathy, with diffuse glomerular capillary congestion, swollen endothelial cells, and near complete obliteration of the peripheral capillary lumina along with the presence of fibrin thrombi (Figure 7). On post-operative day 3 there was evidence of progressive tubular injury with extensive acute tubular necrosis, but additional features of TMA including mesangiolysis were not observed. C4d was negative at both time points (Figure 7), as well as IgM, IgG, IgA, C1q, and C3 (Figures 8 and 9). Wedge biopsies from study termination demonstrated no evidence of cortical necrosis or interstitial hemorrhage and glomerular capillary congestion was no longer diffuse (data not shown). Post-termination analysis of renal tissue confirmed expression of the human transgenes within the porcine kidney parenchyma (Figure S11).

Decedent blood samples were tested daily for the presence of porcine endogenous retroviruses and remained negative (Figure 10). In addition, chimerism, as measured by the presence of expression of the gene for a porcine large ribosomal protein (pRPL4), was not observed at any time point (Figure 10).

4 | DISCUSSION

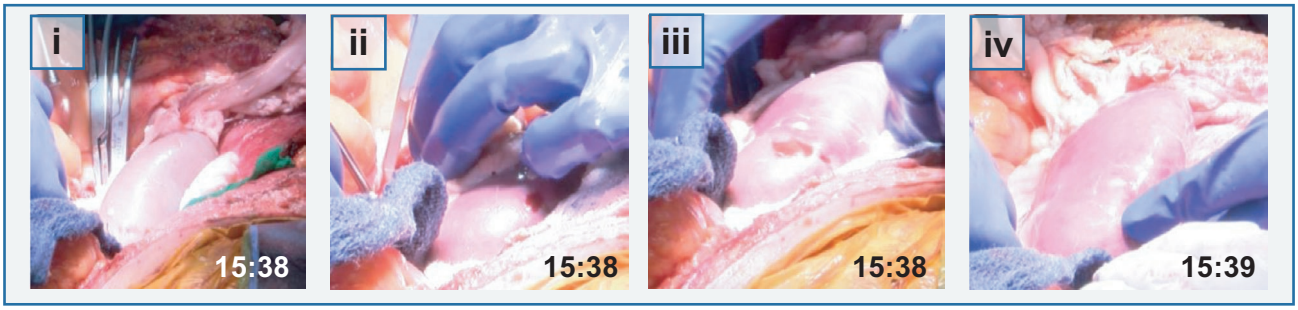
Xenotransplantation is arguably the most pragmatic solution to the organ shortage crisis, but safety and efficacy concerns have limited advancement into humans. In preparation for a phase I clinical trial of porcine renal xenotransplantation at the University of Alabama at Birmingham, we asked what gaps in knowledge must be filled before such a clinical trial could be ethically offered to research subjects. We thus aimed to develop a human preclinical model which would permit the *in vivo* evaluation of critical safety and feasibility tenets of the pig-to-NHP model without risk to a living human. Our study was designed to test five central questions: (1) Is the current suite of porcine genetic modifications sufficient to avoid hyperacute rejection in humans? (2) Would prospective flow-based crossmatching correlate with graft survival free of hyperacute rejection? (3) Would life-threatening intraoperative complications occur during a renal porcine xenotransplant? (4) Would porcine cells and/or pathogens be detected in the blood of a human recipient? (5) Could porcine renal xenotransplantation be safely performed under the conditions necessary for a clinical trial? To this end, we designed and performed this experiment under clinical-grade conditions which included the transplantation of 10-GE porcine kidneys designed specifically for human transplantation into the conventional anatomic position using processes and facilities in compliance with multiple regulatory agencies.

Similar to NHPs, hyperacute rejection was not observed in this human decedent, providing critical evidence that knockout of the genes encoding enzymes that synthesize carbohydrate xenoantigens (i.e., *GGTA1*, *β4GALNT2*, *CMAH*) is indeed sufficient to prevent hyperacute rejection from this mechanism in humans. Importantly, our study addressed a second potential mechanism of hyperacute

FIGURE 3 Reperfusion of porcine renal xenotransplants in the human decedent. Intraoperative photographs demonstrate viable kidney transplants bilaterally. (A) Reperfusion of the right kidney as shown over the course of approximately 1 min. (i) Appearance of the right kidney immediately prior to reperfusion after completion of the vascular anastomosis. Vascular clamps are present in the operative field. (ii) Appearance of the right kidney immediately after removal of vascular clamps. Note darker pink color of the kidney and the appearance of blood on the kidney surface under surgeon's hand. (iii) Appearance of the right kidney 5–10 s after removal of clamps. Reperfusion is progressing from superior to inferior pole. (iv) Appearance of the right kidney 1 min after removal of clamps. Entirety of kidney is now re-perfused. (B) Sequential urine output after reperfusion of the right kidney is shown. Right kidney is depicted by black arrowheads. (i) and (ii) showcase urine output prior to ureteral anastomosis. Right ureter is being held in the surgeon's hand alongside collection cup. Note increased volume of urine in the cup between (i) and (ii). (iii) Urine output from the right kidney after anastomosis to the decedent bladder. Total volume in the collecting Foley bag is shown. (C) Comparable kinetics of reperfusion and absence of hyperacute rejection for the left porcine renal xenograft. (D) Reperfusion biopsy results of the left kidney. The investigators' elected to not perform a reperfusion biopsy of the right kidney given the extremely delicate nature of the porcine xenografts. There was no difference in gross appearance of the kidneys at the time of biopsy

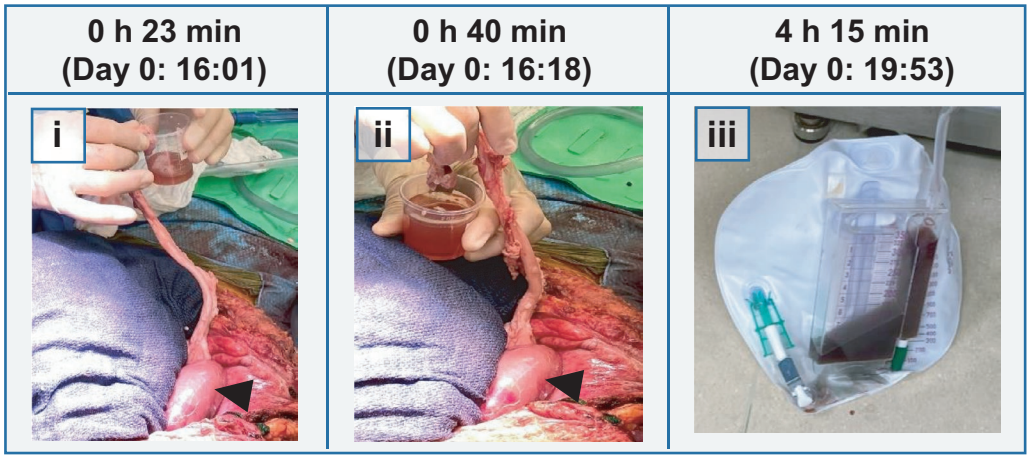
(A)

Right Porcine Renal Xenograft Reperfusion



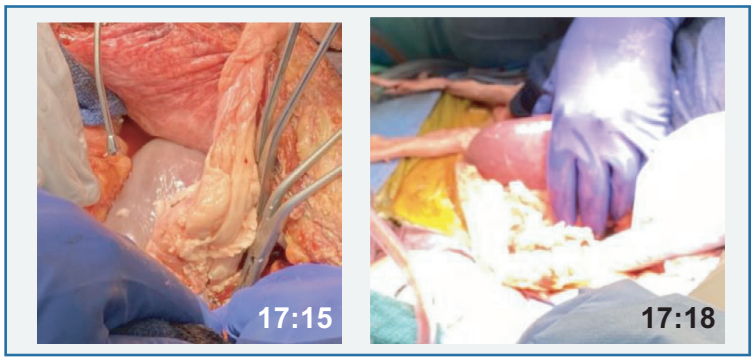
(B)

Right Porcine Renal Xenograft Urine Output



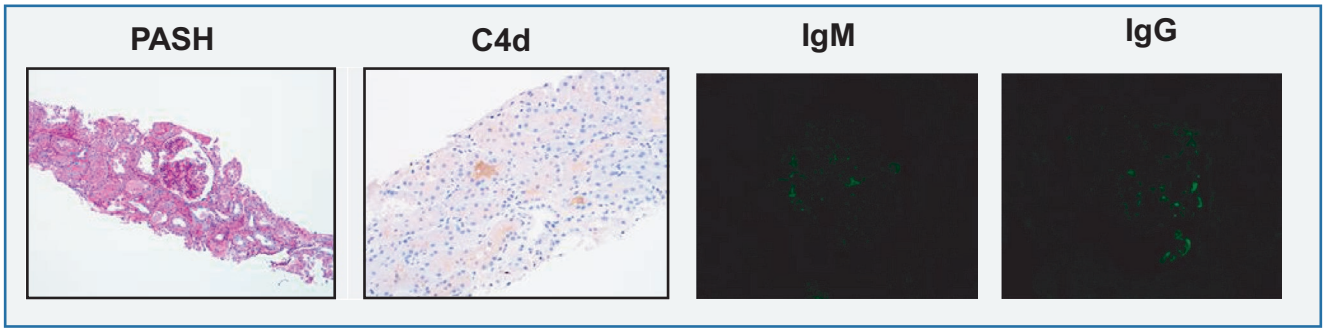
(C)

Left Porcine Renal Xenograft Reperfusion



(D)

Left Porcine Renal Xenograft Post-Reperfusion Biopsy



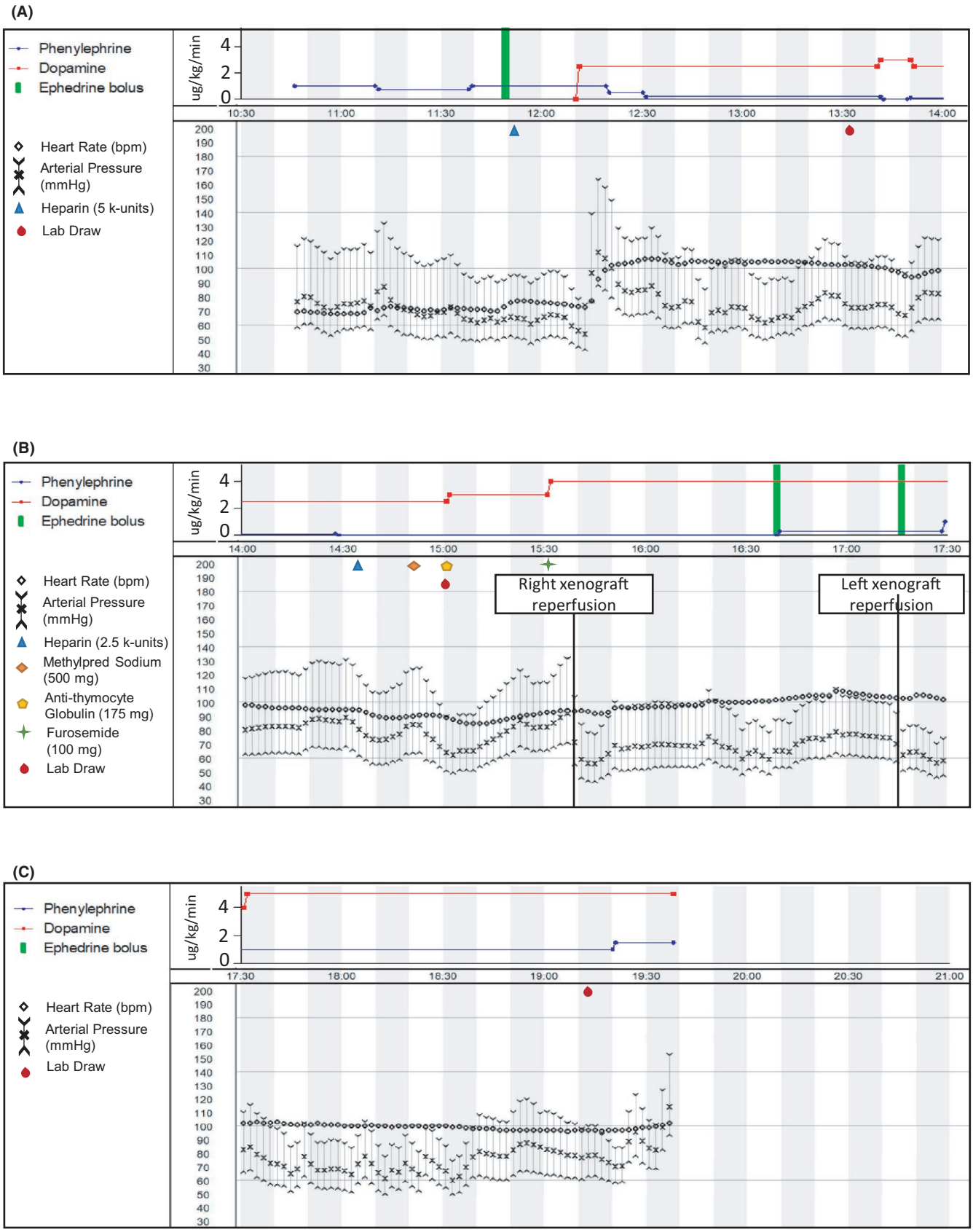


FIGURE 4 Annotated anesthesia report of intraoperative hemodynamic monitoring. Results demonstrate stability of the decedent during bilateral native nephrectomies and transplantation of bilateral kidney xenografts. Phenylephrine and dopamine dosing are shown as continuous infusions while ephedrine was administered as 10 mg boluses. (A) Anesthetic record from 10:30 to 14:00. During this time frame, the decedent underwent native nephrectomies and the xenografts were prepared on the backbench. (B) Anesthetic record from 14:00 to 17:30. Anastomosis and reperfusion of the xenografts is performed. Specific timing of xenograft reperfusions are shown. (C) Anesthetic record from 17:30 to completion of surgery. Ureteral anastomoses were performed during this time frame

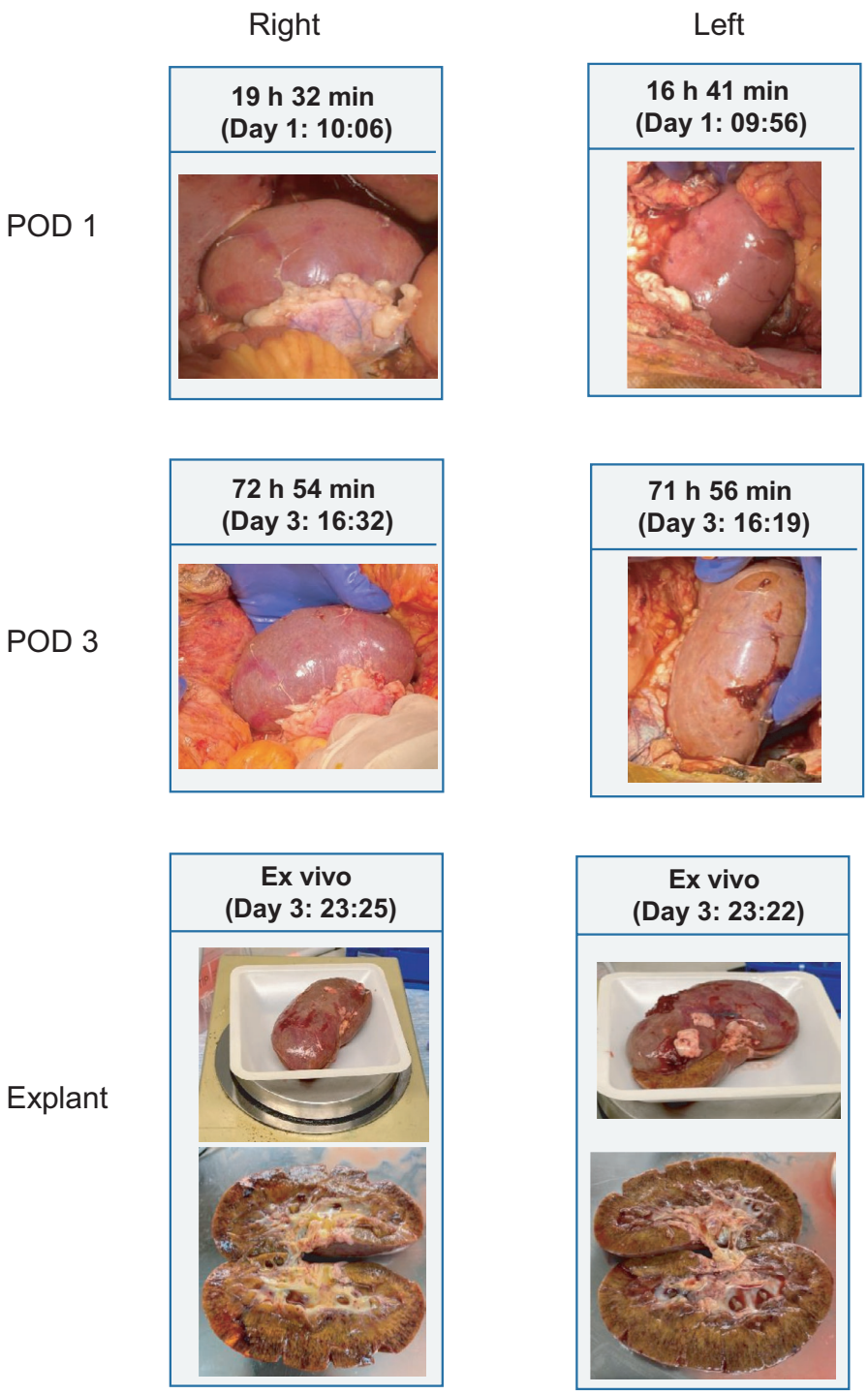


FIGURE 5 Longitudinal assessment of the porcine renal xenografts. Photographs from post-operative days 1 and 3 (POD 1, POD 3) were taken intraoperatively while the kidneys were *in vivo*. Minor blood accumulation underneath the right kidney capsule on POD 1 occurred after biopsy was taken. Yellow tinge of left kidney on POD 3 likely reflects bilirubin staining given hyperbilirubinemia in the decedent

rejection in humans, which is preformed antibody against either the major histocompatibility complex in pigs (swine leukocyte antigen; SLA) or other unknown minor antigens. Although humans are not expected to possess anti-SLA antibody due to prior sensitization events, pre-existing anti-HLA antibody may cross-react with SLA alleles,[17-20] particularly the class II loci, given the sequence homology between pig and human DR, DP, and DQ antigens.[21-23] To this end, we developed and tested a novel flow crossmatch assay which prospectively predicted that hyperacute rejection would not occur. Although preliminary testing of this assay suggested that either fresh or frozen pig PBMCs could be used at the time of crossmatching with a potential recipient (data not shown), we validated these results during this decedent experiment. Although fluorescence intensities varied between fresh and frozen porcine PBMCs, the results overall were internally consistent and easily interpretable given the use of appropriate positive and negative controls. Additional reagent development (i.e., SLA single antigen beads) will be necessary to characterize the antibody specificities of our positive control sera, aid in the interpretation of positive crossmatches, and identify additional potential antigenic targets.

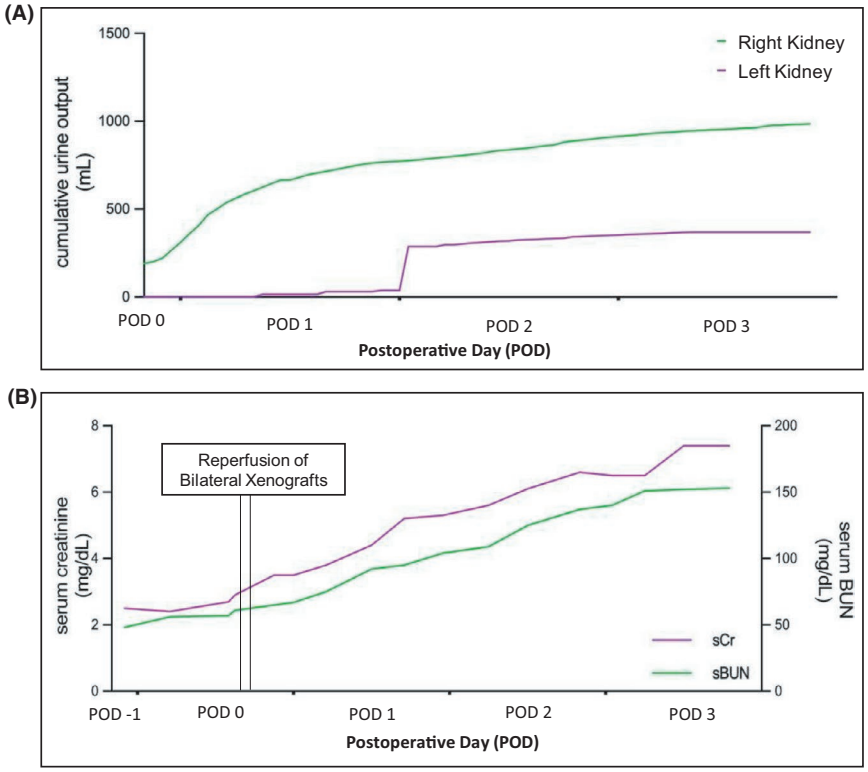


FIGURE 6 Porcine renal xenotransplant function in the human decedent. (A) Cumulative posttransplant urine output from transplantation to study end from right and left xenografts. (B) BUN and creatinine in the decedent's serum. Results prior to POD 0 reflect function of decedent's native kidneys prior to native nephrectomies

TABLE 6 Urinalysis results from right kidney

Urinalysis	POD 1
Color	Red
Clarity	Slightly cloudy
Specific Gravity	1.009
pH	6.0
Protein	2+
Glucose	1+
Ketones	Negative
Blood	3+
Nitrite	Negative
Leukocyte Esterase	Negative
RBC	>25
WBC	0–5

TABLE 7 Results of 24-h urine collection (right kidney)

24 h urine collection	POD 1
Albumin	887.5
Calcium	27.3
Potassium	7
Sodium	95
Osmolality	322
Phosphorus	<10
Urea	630
Creatinine	49

A number of safety goals of this study revolved around the consequences of connecting the circulation of a human with a porcine kidney. Notably, the blood pressures of both a pig and a non-human primate are significantly less than a human, and we tested the assumption that a porcine kidney could withstand the non-trivial increase in human blood pressure. Reassuringly, xenograft vascular integrity was maintained at human mean arterial pressures. Equally important was the relative hemodynamic stability of the decedent upon reperfusion, indicating that washout of inflammatory mediators from the xenograft during reperfusion did not provoke cardiovascular collapse. Finally, we found no evidence of porcine endogenous retrovirus transmission or peripheral chimerism in the decedent based on assays developed in our research laboratories. Although these initial results are encouraging, conclusions are limited given the short duration of the experiment and the unknown sensitivity and specificity of these first-generation research assays, despite the ability of PCR assays to detect as few as 100 copies of PERV-C.[24] Refinement of research laboratory assays to approximate those used in clinical laboratories will be necessary to support future clinical trial efforts, because veterinary diagnostic laboratories with clinical-grade porcine viral testing capabilities may not accept human specimens, and hospital clinical laboratories may not be equipped to test for porcine pathogens. Investment in the development of clinical-grade laboratory assays which utilize next generation sequencing technologies will likely increase the sensitivity and specificity of these assays—especially for the PERV-A/C recombinant virus[25]—and provide the foundation for a robust microbiologic safety plan to support a phase I clinical trial. Such goals can

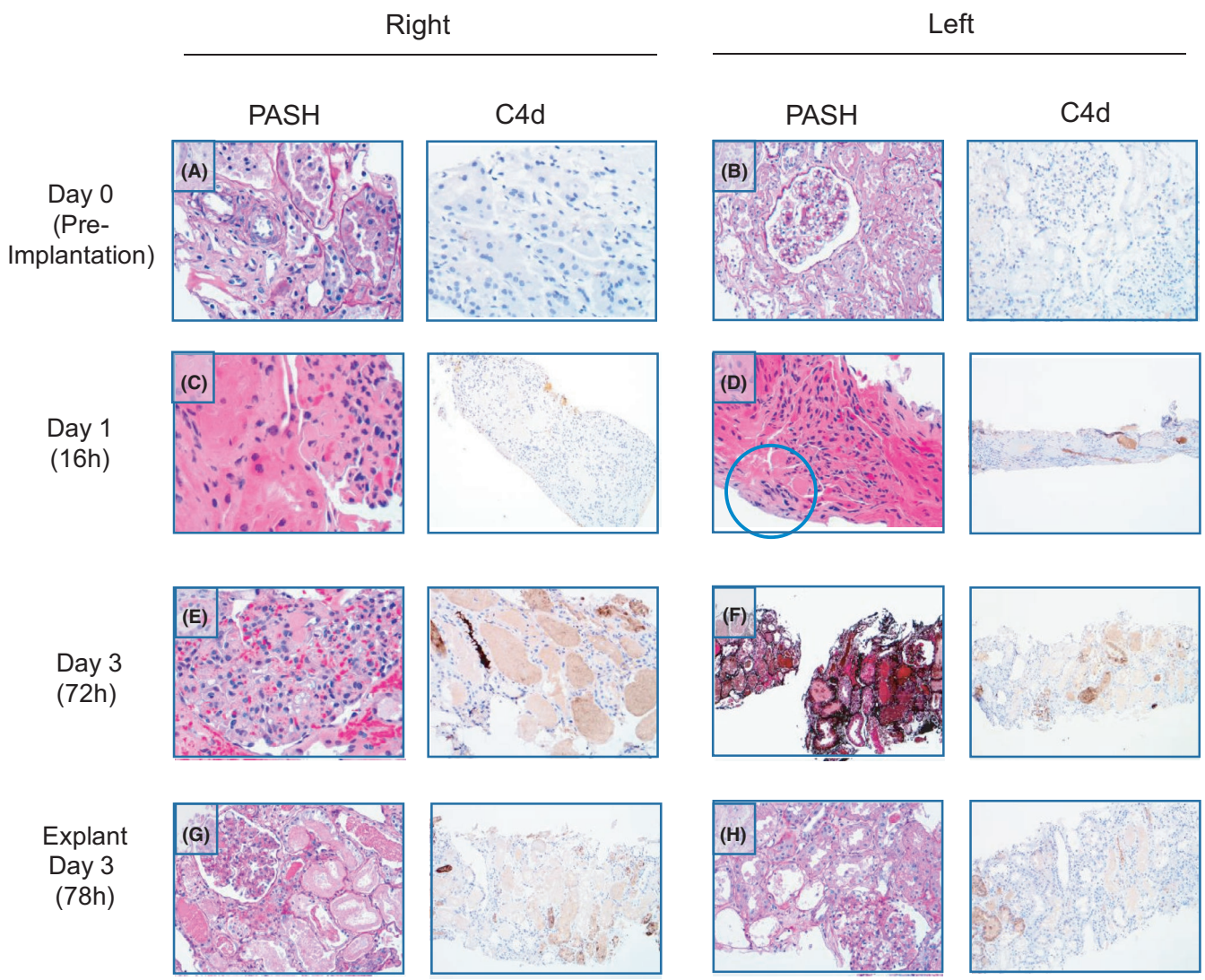


FIGURE 7 Serial histologic examination of the porcine kidney xenografts. All biopsies represent core biopsies. (A, B, G, and H) Were obtained *ex vivo*. (C, D, E and F) Were obtained *in vivo*. Sections are stained with PASH and are 10X, except for (C and D) (40X) and (F) (silver stain). C4d negative throughout. (A and B) Mild to moderate acute tubular injury from cold ischemia. Normal appearance of the capillary network, the mesangium, and the podocytes. (C and D) Glomerulus with multiple fibrin thrombi (blue circle). There is diffuse glomerular capillary congestion with swollen endothelial cells and near complete obliteration of the peripheral capillary lumina. There is presence of fibrin thrombi and fragmented red blood cells consistent with thrombotic microangiopathy (TMA). There is evidence of progressive tubular injury with extensive acute tubular necrosis (ATN). No mesangiolysis is appreciated. (E and F) Glomerular congestion and acute tubular necrosis. Endothelial cells remain segmentally swollen with partially obliterated lumina and rare fibrin thrombi with improvement of glomerular injury. (G and H) Acute tubular injury persists. Glomeruli with segmental endothelial swelling. No fibrin thrombi

be accomplished as evidenced by recent studies published out of New Zealand, where seven-year follow-up data of human subjects transplanted with encapsulated porcine islets indicate no transmission of zoonotic disease.[26,27] These data recapitulate other findings in preclinical animal models demonstrating no *in vivo* transmission of PERV.[28]

Although our study was not designed to optimize renal performance or immunologic outcomes, the decedent model afforded the opportunity to perform serial renal biopsies and assess renal function. Urine output was initially robust from the right kidney but significantly less from the left kidney. While the etiology of this mismatch in renal performance is unknown, the accrual of additional warm ischemic time on the left kidney during clamping in the donor may have played a role. Additional studies will be needed to determine how ischemic time is tolerated by porcine xenografts and what conditions optimize renal preservation for human recipients. On a similar note, we elected to transplant both porcine kidneys into one decedent, as the porcine nephron mass required to support a human is also unknown. The decedent model may yet prove valuable in determining how many porcine kidneys are needed to support a human adult.

Despite the transplantation of both kidneys, serum creatinine did not decrease and neither kidney excreted significant creatinine into the urine. The etiology of this renal dysfunction is unclear and is likely

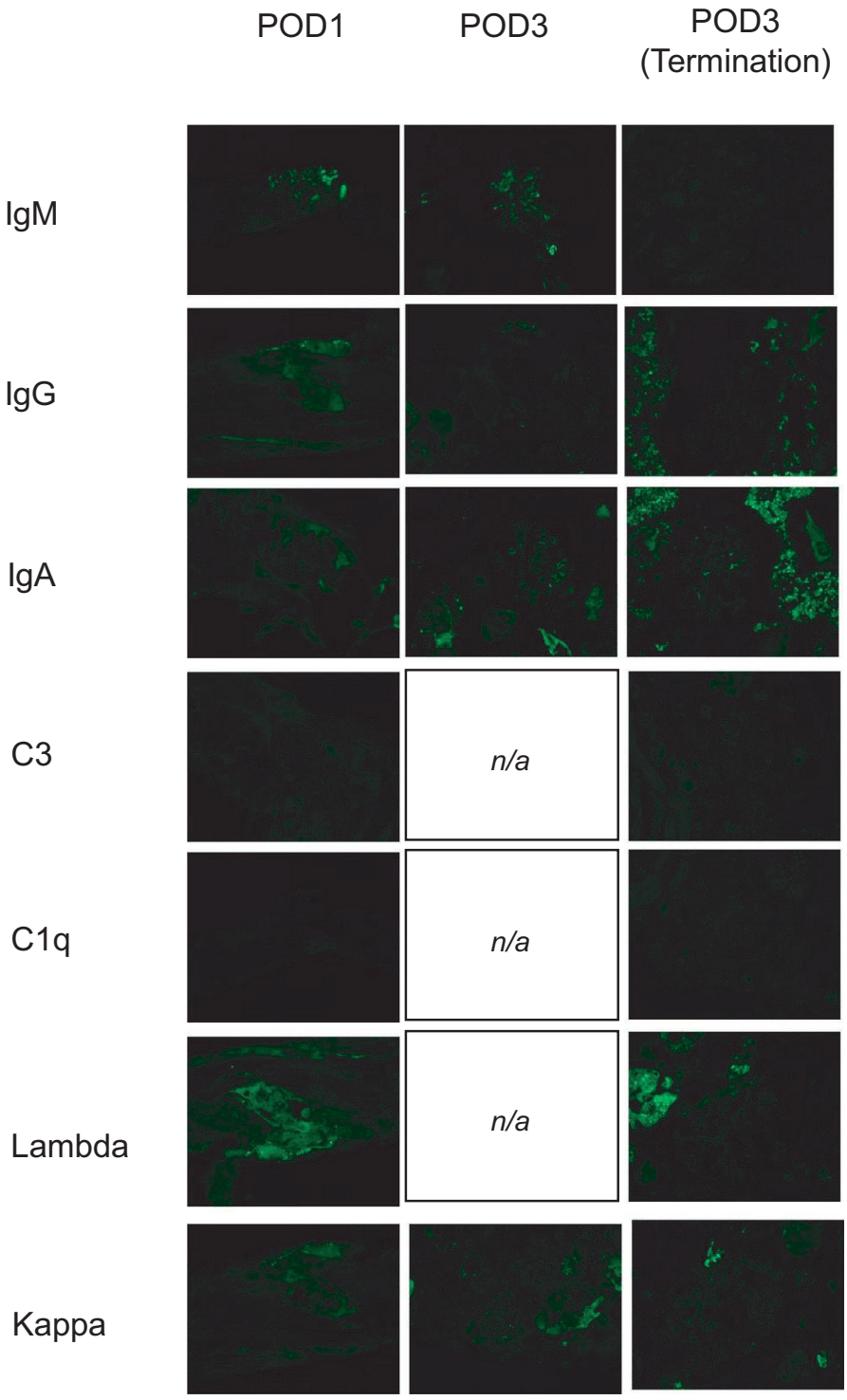


FIGURE 8 Immunofluorescence staining (left xenograft). Core biopsies of the left renal xenograft were obtained, fixed in formalin and paraffin embedded, and then submitted for immunofluorescence microscopy to Arkana Laboratories (Little Rock, AR). Tissues were stained as indicated following protease digestion. POD 1 samples had 2 glomeruli present for evaluation. No glomerular or extraglomerular staining was noted. Kappa and lambda light chains stained equally throughout the tubules and interstitium. On POD 3, two intact glomeruli were evaluated. These glomeruli revealed 1+ IgM staining of the mesangium and segmental capillary loop thought to be due to non-specific entrapment. IgA, IgG, and kappa light chain stains were negative in the glomeruli without significant extraglomerular staining. At termination on POD 3, core biopsies were performed of the explanted xenograft and analyzed. Six glomeruli were present for evaluation, without significant glomerular or extraglomerular staining

multifactorial. Although serum creatinine and BUN were normal in the donor pig and are normal in the 10-GE herd at the UAB XPC (BUN: 30 ± 9.4; creatinine: 0.92 ± 0.24 [avg, SD]; $n = 17$ measurements in 10 animals over 60 days), genetic modifications of these animals clearly altered the overall structural integrity of the renal parenchyma. To the best of our knowledge, such differences in tissue integrity have not been reported in other genetically modified pigs, and it is not yet clear to what degree these structural changes might impact renal function or recovery. Alternatively, poor renal recovery may reflect the deleterious milieu of brain death characterized by complement activation[29] and hemodynamic decompensation requiring vasoactive agents.[15] Finally, renal dysfunction in the decedent may have also been impacted by microvascular injury of unclear etiology. Of note, xenograft histology demonstrated endothelial injury with diffuse TMA on post-operative day 1, but there was no evidence of progression to cortical necrosis or interstitial hemorrhage by post-operative day 3, as might be expected if TMA was due to significant antibody-mediated damage. As the xenograft biopsies were negative for IgM, IgG, C4d,

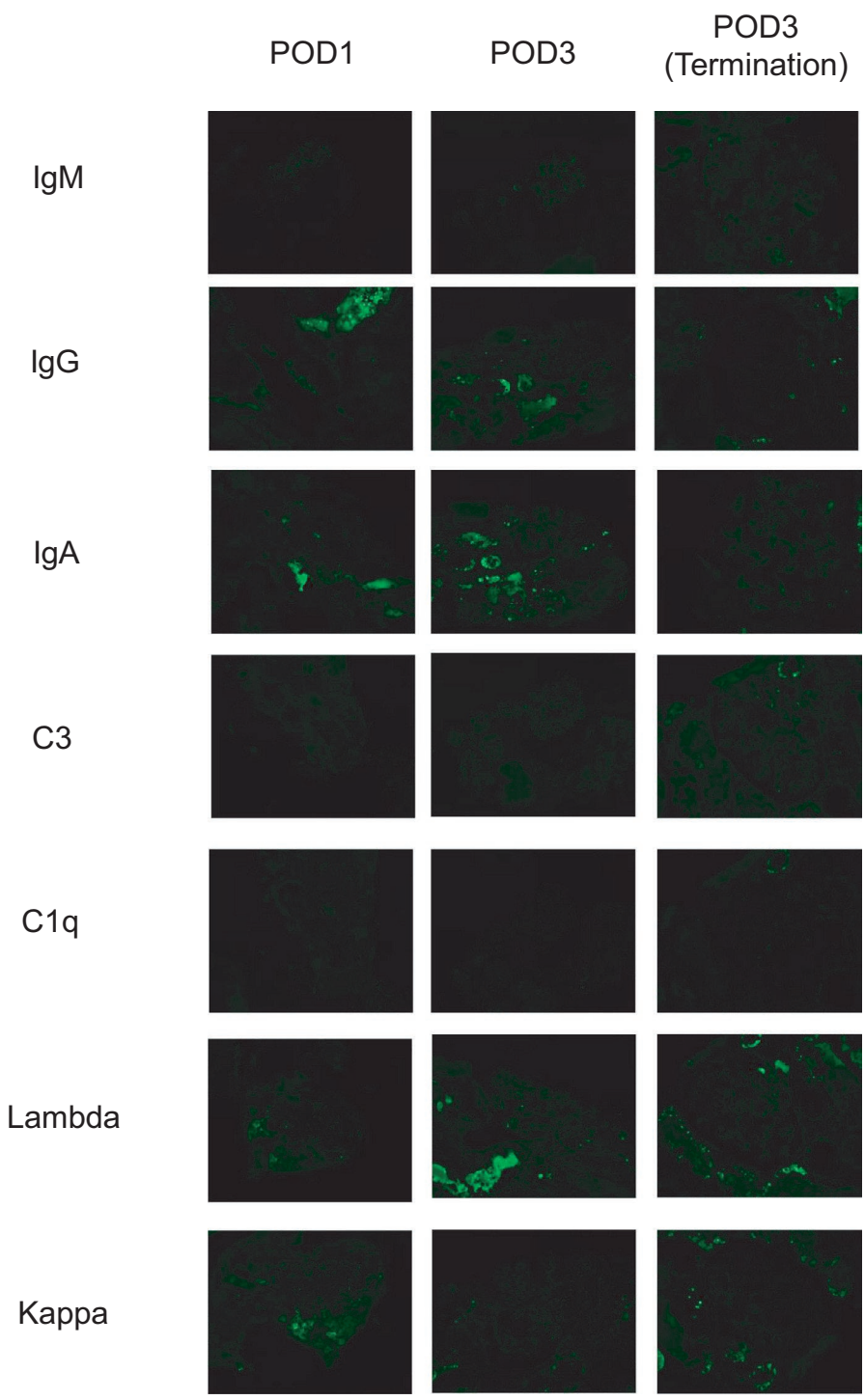


FIGURE 9 Immunofluorescence staining (right xenograft). Core biopsies of the right renal xenograft were obtained, fixed in formalin and paraffin embedded, and then submitted for immunofluorescence microscopy to Arkana Laboratories (Little Rock, AR). Tissues were stained as indicated following protease digestion. On POD 1, no significant staining is observed in the glomeruli ($n = 3$). There is also no significant extraglomerular staining, and kappa and lambda staining is homogenous throughout the tubulointerstitium. Similar results were observed on biopsies from POD 3 (8 glomeruli evaluated) and the explant at termination on POD 3 (5 glomeruli evaluated)

C1q, and C3, we must consider the possibility that the TMA is not mediated by complement or antibody in the xenografts and is instead the result of some other unknown mechanism or molecular incompatibility. Nevertheless, the observed TMA may still yet be the result of complement-mediated cytotoxicity,[29] as the alternative complement pathway does not require antibody or C4 to trigger the formation of the membrane attack complex (MAC; C5-9).[16] Moreover, although the 10-GE xenograft has been engineered to contain complement inhibitor genes (decay accelerating factor, DAF; membrane cofactor protein, MCP/CD46) to address some of the histologic findings associated with acute humoral xenograft rejection,[30] these proteins merely slow MAC formation and do not necessarily prevent it.[29] Additional genetic and/or pharmacologic interventions which prevent complement-mediated cytotoxicity may thus be necessary to improve graft survival and function. Of note, an anti-C5 antibody is available[31] and utilized for the treatment of severe antibody-mediated rejection in human allotransplantation,[32,33] and prevention of MAC formation with anti-C5 antibody was recently shown to improve xenograft survival in a NHP model.[34] Collectively, these findings highlight the need for ongoing work in this area and suggest that both NHP and human studies may

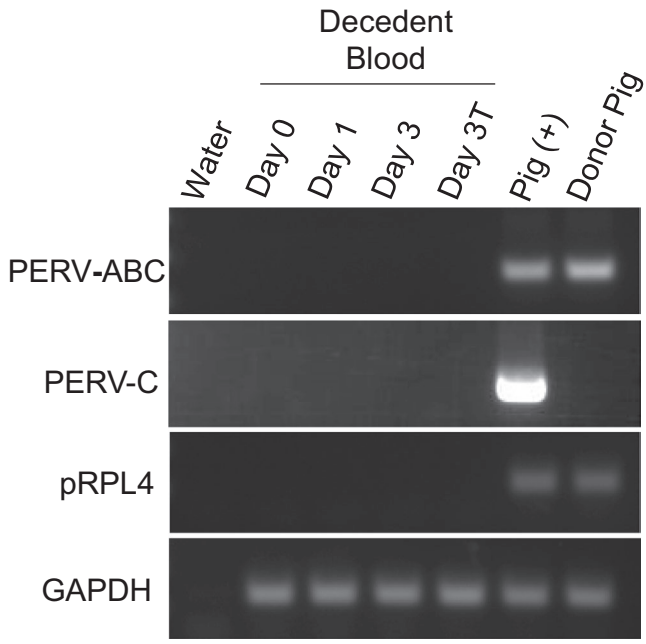


FIGURE 10 Longitudinal analysis of porcine endogenous retrovirus transmission and microchimerism in the decedent. No PERV or microchimerism (pig-specific RPL4) was detected by RT-PCR using mRNA from different time intervals posttransplant. Pig(+) is a PERVC-positive pig control. GAPDH is an endogenous control showing presence of mRNA in all samples. Water is shown as a negative control

be necessary to understand the molecular basis and clinical implications of these biopsy findings.

In conclusion, we addressed critical safety and feasibility questions in xenotransplantation by using a novel pre-clinical human model under significant regulatory oversight. Our results add significantly to the prior knowledge generated in non-human primate models and suggest that many barriers to xenotransplantation in humans have indeed been surmounted.[35] Importantly, the decedent model identified numerous areas where additional understanding is needed, and all of our results must be interpreted cautiously within the numerous limitations of the model. Whether the new knowledge and process gaps identified by our study can be addressed using the decedent model or a combination of *in vitro* studies with preclinical animal models and even clinical trial data in living humans remains to be determined. Nevertheless, the decedent model has significant potential to propel not only the field of xenotransplantation forward but to answer a multitude of other scientific questions unique to the human condition.

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DISCLOSURE

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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