Supplementary Appendix

Supplement to: Griffith BP, Goerlich CE, Singh AK, et al. Genetically modified porcine-to-human cardiac xenotransplantation. N Engl J Med:35-44. DOI: 10.1056/NEJMoa2201422

This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Materials

In Reference to: Genetically Modified Porcine-to-Human Cardiac Xenotransplantation

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Supplemental Discussion:

After four iterative changes to the application and two conference calls with the FDA over 10 days, we received approval on December 31st, 2021. The approval required us to affirm that the eIND "would not interfere with the conduct of future clinical trials required by an IND", and to obtain approval from our Institutional Review Board (IRB).

Our patient was disqualified from other advanced therapies principally because of his history of non-compliance. He had been non-ambulatory for 8 weeks prior to experimental xenotransplantation. His level of preoperative deconditioning was further compounded by his cross clamp-related Type A aortic dissection, perioperative acute renal failure and bacterial peritonitis discovered on POD 10, which resulted in prolonged postoperative physical rehabilitation and antibiotic needs. After successfully weaning from ECMO shortly after transplantation, he participated in active rehabilitation for almost 2 months. However, several barriers to full improvement of his clinical status ensued in his postoperative course.

The patient had a history of mild chronic thrombocytopenia with further decline in his counts (<20,000 cells/μL) postoperatively. Preliminary autopsy found a normal spleen without evidence of sequestration of platelets. Preoperative leukopenia, presumably from antibiotics, worsened postoperatively and responded to GCSF. Leukopenia and thrombocytopenia affected decision making regarding induction immunosuppression as well, which deviated from our NHP experience.[1,2] A single, rather than double, dose of Rituximab was given just prior to transplantation. It adequately suppressed peripheral circulating CD20+ cells. However, a lymph node sample at the time of chest closure POD 2 showed residual CD20+ B cells and guided an additional dose on POD 8 to target lymph-bearing tissue. The usual total preoperative induction dose of 10 mg/kg ATG was substituted for a 4 mg/kg graduated dose between POD 1-4. A repeated single dose KPL-404 dosing scheme was utilized to target appropriate drug levels. Pharmacokinetics may have also been complicated by resuscitation, volume shifts and continuous renal replacement therapy (CRRT). The impact of withdrawal of MMF at POD 24 should also be considered regarding the overall immunosuppression of the recipient.

Postoperative monitoring of both the xenograft and for zoonoses were performed using traditional clinical measures and included novel highly sensitive cfDNA assays. Longitudinal xenograft-specific antibody assays were also used to aid in detecting elicited donor-specific antibody responses. These results were generally delayed by 1 week, as they are dependent on growing cell cultures of the source animal's aortic endothelial cells, incubating these cells with recipient serum and analyzing antibody binding by flow cytometry. Point-of-care troponin I testing was most useful to detecting early xenograft injury largely because the data were instantaneous and previously validated by our NHP work.[1–3]

The suddenness and severity of acute diastolic heart failure in our patient had not been anticipated from our NHP studies.[1,2] It is unclear whether there would be improvement

of diastolic dysfunction and myocardial thickening with prolongation of ECMO support. Presumably, the healing would require elimination of edema without subsequent fibrotic healing. The preservation of systolic function is of interest and gives credence to the possibility of reversibility. No evidence of pathology outside of the heart related to the xenotransplantation and associated therapeutics were observed on preliminary autopsy.

In a single report, IVIG has not shown to have complement dependent cytotoxicity to pig xenogeneic tissues in vitro or in vivo, however, authors cautioned that specific doses given to a patient should exhibit minimal xenograft specific antibody-mediated cytotoxicity prior to adminstration.[4] Our analysis retrospectively confirmed some PAEC binding, but lack of complement-mediated cytoxicity.

In this emerging field, a multi-faceted approach to surveillance is important, which includes rapid determination of anti-porcine antibodies and immunosuppressive monitoring. Biomarkers will include troponin I and xd-cfDNA. In parallel with echocardiography, these are likely useful for determining the state of the xenograft (Figure 3a-c). More clarity in the pathophysiology of the acute diastolic failure will undoubtably help define antecedent markers on early endomyocardial biopsy as well. More sensitive screening assays may be required for pig source animals to better assess latent viruses such as pCMV.

Based on our experience, we believe there are morbidly ill patients with heart failure who do not meet criteria for advanced therapeutic options, who may choose xenotransplantation. Once our patient knew that he was unlikely to survive with continued medical therapy, he readily accepted this experimental procedure. His son stated, “We hope this story can be the beginning of hope and not the end. We also hope that what was learned from his surgery will benefit future patients and hopefully one day, end the organ shortage that costs so many lives each year.”[5]

Supplemental Methods:

Immunosuppression and Monitoring: The goal was to replicate our successful laboratory protocols in non-human primate (NHP) while balancing preoperative leukopenia (2,200-3,200 cells/μL) and baseline thrombocytopenia (90-111,000 cells/μL) of the patient prior to induction (Table S2 and Figure S1).[1,2] Rituximab and anti-thymocyte globulin (ATG) were used for B and T cell depletion, respectively, and complement C1 esterase inhibitor (Berinert, King of Prussia, PA) was used for complement inhibition. Humanized monoclonal antibody to block CD40 co-stimulation (KPL-404, Kiniksa Pharmaceuticals, Ltd., Hamilton, Bermuda), was administered using repeated single dosing. A pulse-dose of methylprednisolone (1,000mg, day of xenotransplant) was also administered.[1] Maintenance immunosuppression included mycophenolate mofetil (MMF), KPL-404 and a rapid taper of methylprednisolone (125mg to 30 mg daily). We monitored peripheral blood mononuclear cells (PBMCs) by flow cytometry for B ($CD20^+$) and T ($CD3^+$) lymphocytes. KPL-404 levels were monitored at pre-dose trough, peak, and longitudinally over time (with an assay developed and performed by Kiniksa Pharmaceuticals, Ltd.). Prior to transplantation, donor-specific IgM and IgG antibody levels were acceptably low.

Serial transthoracic echocardiography (TTE) using the Phillips EPIQ CVx Ultrasound system and X5-1 transducer was performed at least twice a week to closely monitor the xenograft. Endomyocardial biopsies (embx) of the right ventricle and right heart catheterization for pressures were planned for routine surveillance and for cause, based on clinical status. We performed histology with Hemotoxin & Eosin (H&E) stains and immunohistochemistry (IHC) (CD3, CD20, CD68, CD3d and CD4d) on embx specimens.

Serum levels of troponin I were followed longitudinally. Xenograft-derived cell free DNA (xd-cfDNA) were drawn weekly and determined off-site by CareDx (Brisbane, CA).

Infection Control Plan: Key aspects of our infection control plan included use of enhanced contact precautions, use of disposable instruments when feasible, and sequestering of non-disposable instruments following enzymatic/bleach cleaning and sterilization. Healthcare providers and laboratory personnel were instructed to follow prion precautions when transporting and processing clinical samples. A series of educational sessions were held and healthcare workers were given the opportunity to opt in or opt out of providing care to the xenorecipient. All providers entering the patient's room signed a log to allow for contact tracing. We developed a passive surveillance program in which select healthcare providers underwent blood draws at pre-defined timepoints for pathogen surveillance.

Donor Specific Antibody (DSA) Assay: Heat inactivated recipient serum and healthy subjects known to have high (High Human) and low (Low Human) levels of DSA antibodies, served as positive controls. Serum was incubated with 10GE source animal aortic endothelial cells (pAEC) for 2 hrs at 4°C. After incubation, pAEC were washed twice with phosphate-buffered saline (PBS) and non-specific protein-binding sites were

blocked with 10% normal goat serum (Abcam, MA, USA) for 20 min at 4°C. Then, fluorescence conjugated (FITC or Alexa-Fluor 488) goat anti-human IgM and IgG (Jackson ImmunoResearch Laboratories, PA, USA) was added (1:100 final concentration in PBS) and allowed to incubate for 60 min at 4°C. After incubation, the pAECs were washed twice with PBS and resuspended to perform Flow Cytometry (Cytek Aurora or BD Accuri C6 Plus, CA, USA). Ten thousand events per sample were counted and samples were analyzed using FlowJo software (FlowJo LLC, OR, USA).

Preservation of the Xenograft: The pig heart was procured from the source animal in our laboratory clinical-grade operating room.[6] Non-ischemic perfusion of the 328 gm pig heart using the XVIVO system lasted 114 minutes. Perfusate was cooled to 8 degrees centigrade. It consisted of 4 parts Steen cardiac solution mixed with one part human blood which was type-matched to the recipient.[7–9] Perfusion was fixed at 20mmHg in the aortic root. Flow increased from 148 cc/min to 194 cc/min suggesting coronary relaxation. Total cold ischemia time was 150 minutes. The implant required 63 minutes and was interrupted three times for intermittent cardioplegia with XVIVO perfusate harvested from the circuit. An additional circulatory arrest time of 13 minutes was required after initial cardiac reanimation for repair of a Type A aortic dissection caused by the aortic cross clamp. After both the first and second circulatory arrest and rewarming, the heart began beating spontaneously with only temporary need of epicardial pacing.

Surgical Technique: The XVIVO system was transported into the hospital's operating room from the laboratory operating room. The redo sternotomy in the anesthetized patient was delayed until the donor heart was delivered. Implantation was performed using a biatrial anastomosis to accommodate size mismatch of the relatively smaller donor heart. The host's atrial circumferences were reduced, and the considerable mismatch of interatrial distance was accounted for using asymmetric suturing. Following the release of the aortic cross clamp, a dissection flap was noted in the descending aorta on transesophageal echocardiography (TEE). An intravascular ultrasound confirmed a compressed true lumen throughout the course of the aorta from the area of the previous cross clamp to the bifurcation of the abdominal aorta and right femoral artery. This necessitated an ascending aortic repair with 13 minutes of circulatory arrest. A thoracic endograft substantially restored the true lumen

Figure S1:

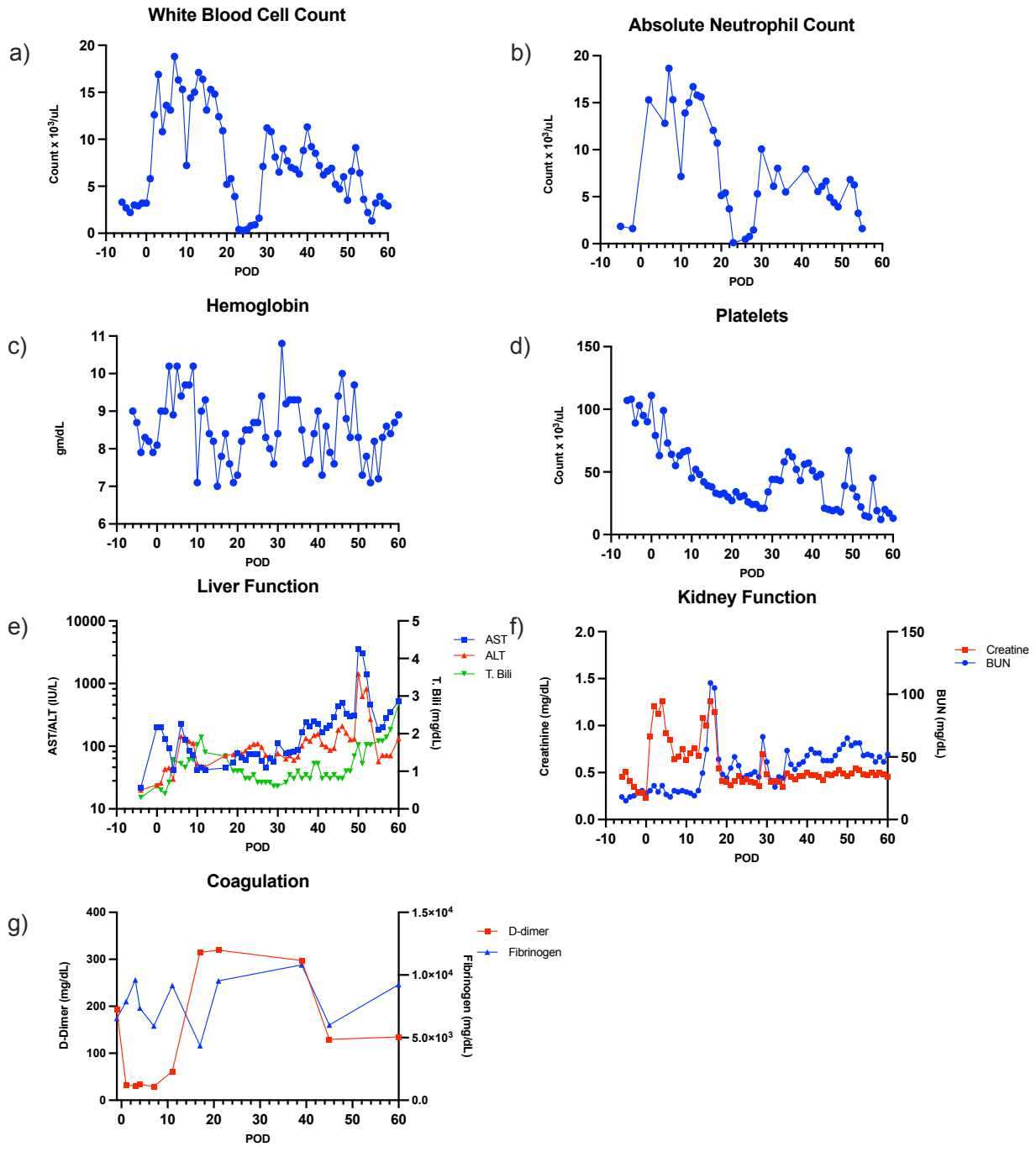

Figure S1: Laboratory parameters before and after xenotransplantation. AST= aspartate aminotransferase, ALT= alanine aminotransferase, BUN=blood urea nitrogen.

Figure S2:

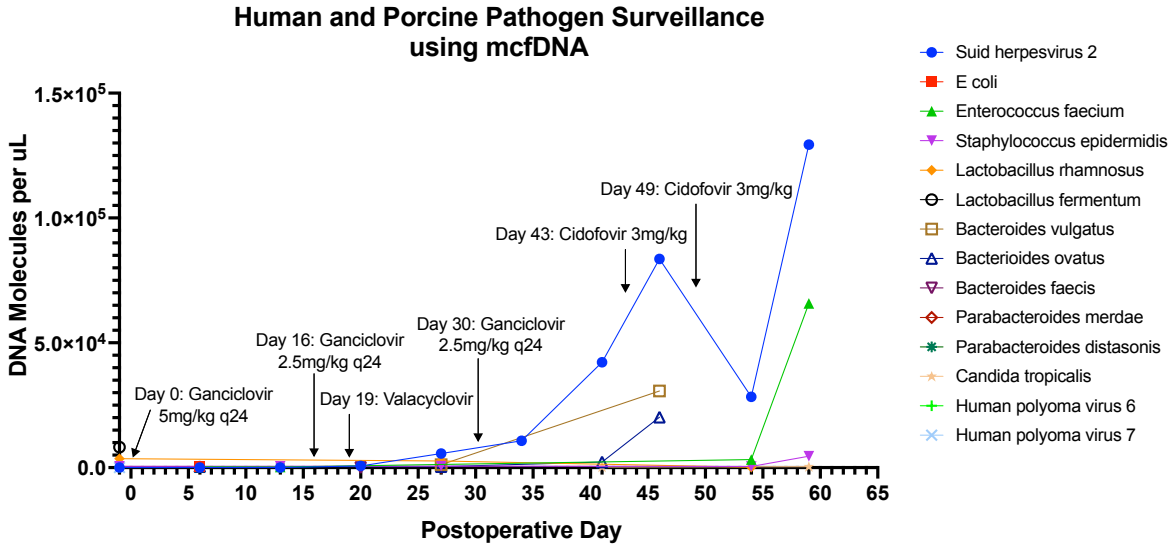

Figure S2: Unbiased longitudinal surveillance of recipient plasma by mcfDNA revealed presence of suid herpesvirus 2 (porcine cytomegalovirus, pCMV). Superimposed treatment for pCMV is indicated by arrows. There was no detection of latent human DNA viruses following xenotransplantation.

Table S1: Genetic Modifications of Source Animal for Cardiac Xenotransplantation		
Genetic Modification	**Mechanisms**	**Properties**
Xenogeneic Carbohydrate Knockout		
Galactose-α-1,3-galactose KO (GTKO)	Deletion of immunogenic Galactose-α-1,3-galactose (Gal) glycan through knockout of the synthetic enzyme alpha1,3-galactosyltransferase (GT)	Anti-Immunogenic
β1,4-N-acetylgalactosyltransferase KO (B4GalKO)	Deletion of immunogenic blood group SDa antigen through knockout of the synthetic enzyme (B4GalNT2)	
CMP-N-acetylneuraminic acid hydroxylase KO (CMAHKO)	Deletion of immunogenic glycan N-glycolylneuraminic acid (Neu5Gc) through knockout of the synthetic enzyme CMP-N-acetylneuraminic acid hydroxylase (CMAH)	
Growth Hormone Receptor Knockout (GHRKO)	Reduction of downstream insulin growth factor-1 (IGF-1) signaling	Reduce intrinsic graft growth
Human Transgene Expression		
CD46	Suppress human complement activity by mediating cleavage of C3b and C4b complement deposition	Complement Regulation
Decay Accelerating Factor (DAF)	Inhibits C3 and C5 convertase enzymes and downstream complement activation	
Endothelial Cell Protein C Receptor (EPCR)	Activates Protein C	Anti-Coagulation
Thrombomodulin (TBM)	Binds human thrombin, and activates Protein C via activated thrombin	
Hemeoxygenase-1 (HO-1)	Decreases oxidative products	Anti-Inflammatory
CD47	Interacts with macrophage signal regulatory protein (SIRP)α to prevent opsonization and phagocytosis of xenogeneic tissue	

Table S2: Induction Immunosuppression				
	Date	**Day**	**Drugs**	**Dose**
Induction:	**1/6/22**	-1	Methylprednisolone	125 mg
			Rituximab (brand)	375 mg/m^2 (dose: 800 mg, rounded to nearest 100 mg per protocol)
			Berinert (C1 esterase inhibitor)	20 U/Kg (dose: 1500 units, rounded to nearest vial size)
			KPL-404*	-
Day of Xenotransplantation:	**1/7/22**	At incision	Methylprednisolone	1000 mg
			Berinert (C1 esterase inhibitor)	20 U/Kg (1500 units, rounded to nearest vial size)
		After Hemostasis	KPL-404	-
		After Multiple Blood Products, Including FFP	Berinert (C1 esterase inhibitor)	20 U/Kg (1500 units, rounded to nearest vial size)
Postoperative Day (POD) #1:	**1/8/22**	1	Methylprednisolone	125 mg IV
			anti-thymocyte globulin (ATG)	1 mg/Kg (75 mg, dosed off IBW)

			MMF	500mg BID IV
Postoperative Day (POD) #2:	**1/9/22**	2	Methylprednisolone	60 mg IV (just use pre-medication from standard steroid taper)
			anti-thymocyte globulin (ATG)	1 mg/Kg (75 mg, dosed off IBW)
			MMF	500mg BID IV
Postoperative Day (POD) #3:	**1/10/22**	3	Methylprednisolone	60 mg IV (just use pre-medication from standard steroid taper)
			anti-thymocyte globulin (ATG)	2 mg/Kg (150 mg, dosed off IBW)
			MMF	500mg BID IV
Postoperative Day (POD) #7:	**1/14/22**	7	Methylprednisolone	40 mg IV (just use pre-medication from standard steroid taper)
			MMF	500mg BID IV
Postoperative Day (POD) #8:	**1/15/22**	8	Methylprednisolone	40 mg IV (just use pre-medication from standard steroid taper)
			Rituximab (brand)	375 mg/m^2 (dose: 800 mg, rounded to nearest 100 mg per protocol)
			MMF	500mg BID IV
* Subsequent administrations of KPL-404 were based on repeated single dosing to target appropriate drug levels.				

Table S3: Results of Commercial Pathogen Screening of Source Pig				
Test	**Sample**	**Assay**	**Result**	**Date**
Hepatitis E	Feces	Real-time PCR	Negative	1/7/22
Herpes virus gamma	Buffy coat	PCR	Negative	1/7/22
Influenza A	Nasal swab	Real-time PCR	Negative	1/7/22
Mycoplasma hyopneumoniae	Nasal swab	Real-time PCR	Negative	1/7/22
Porcine cytomegalovirus	Nasal swab	Real-time PCR	Negative	1/7/22
Porcine circovirus type 2	Serum	Real-time PCR	Negative	1/7/22
Porcine circovirus 3	Serum	Real-time PCR	Suspect [Ct 39]	1/7/22
Porcine Epidemic Diarrhea virus (S gene)	Feces	Real-time PCR	Negative	1/7/22
Porcine deltacoronavirus	Feces	Real-time PCR	Negative	1/7/22
Transmissible Gastroenteritis virus	Feces	Real-time PCR	Negative	1/7/22
Porcine reproductive and respiratory syndrome virus (PRRSV)	Serum	Real-time PCR	Negative	1/7/22
Porcine endogenous retrovirus A	Buffy coat	PCR	Positive [Ct 20]	12/21/21
Porcine endogenous retrovirus B	Buffy coat	PCR	Positive [Ct 22]	12/21/21
Porcine endogenous retrovirus C	Buffy coat	PCR	Negative	12/21/21

Table S4: KT® Research Use Only Pathogen screening by mcfDNA	
Virus	Sequences**
Porcine type-C oncovirus	1
Suid herpesvirus 2 (porcine CMV)	1
Porcine parvovirus*	1
Porcine circovirus 1 (PCV1)*	64
Porcine circovirus 2 (PCV2)*	2423
Porcine circovirus 3 (PCV3)*	251
Porcine circovirus 4 (PCV4)*	1
African swine fever virus	133
Suid alphaherpesvirus 1	3
Porcine lymphotropic herpesvirus 1	1
Porcine lymphotropic herpesvirus 2	1
Porcine lymphotropic herpesvirus 3	1
*single-stranded DNA virus **source: National Center for Biotechnology Innovation (NCBI) RefSeq and Genbank	

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