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Orthotope kardiale Xenotransplantation

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Langzeitergebnisse von präklinischen Versuchen

vorgelegt von

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(2021)

Für Kristin,

Grischa, Nike, Clara

und meine lieben Eltern

Margund und Werner

Inhaltsverzeichnis

1. Einleitung

Die allogene Herztransplantation gilt als therapeutischer Goldstandard für die terminale Herzinsuffizienz. Leider ist der Bedarf an Spenderorganen seit vielen Jahren weitaus höher als das Angebot. Im Jahre 2018 wurden in der Eurotransplant-Regionen nur 619 Herztransplantationen durchgeführt; 1132 Patienten waren auf der Warteliste, wovon 141 Patienten verstarben, weil kein passendes Organ verfügbar war[1]. In den letzten Jahrzehnten wurden vermehrt mechanische Herzunterstützungsverfahren implantiert, um die Wartezeit auf ein passendes Spenderorgan zu überbrücken („bridge to transplant"); in manchen Fällen dienen diese Systeme auch als Alternative zur Herztransplantation („destination therapy"). Auf Grund von hohen Komplikationsraten sind diese jedoch bislang der Transplantation in Bezug auf Lebensqualität und Überlebenszeit unterlegen[2].

Die Xenotransplantation, also die Verpflanzung von speziesfremden Organen, könnte eine alternative Lösung für die bestehende Organknappheit darstellen. Als Spendertiere erscheinen Schweine am besten geeignet zu sein[3]. Trotz vieler physiologischer Gemeinsamkeiten existieren zwischen Mensch und Schwein jedoch wichtige biologische Barrieren. Diese betreffen insbesondere die Aktivierung des angeborenen und adaptiven Immunsystems, die Dysregulation der Komplementaktivierung und die Inkompatibilität der Gerinnungssysteme. Genetische Modifikationen der Spenderschweine können helfen, diese Barrieren zu überwinden: Durch Entfernung des wichtigsten Xenoantigens (Galaktose-α-1,3-Galaktose, αGAL), gegen welches Primaten präformierte Antikörper besitzen, kann die schwere hyperakute Abstoßung verhindert werden[4]; durch Einbringen von humanen Genen (hCD46 und hTBM) können Komplement- und Gerinnungssysteme besser reguliert werden[5]. Wie bei der Allotransplantation ist zusätzlich eine medikamentöse Immunsuppression nötig, um langfristig eine Transplantatabstoßung zu verhindern.

Im heterotop-abdominellen Xenotransplantationsmodell konnte durch Kombination von genetischen Modifikationen der Spendertiere (αGAL-Knockout/hCD46/hTBM) und einer auf Kostimulation basierenden Immunsuppression (anti-CD40-Antikörper, zusätzlich Mykophenolat-Mofetil und Steroide) das Überleben eines Schweineherzens im Pavian von mehr als 900 Tagen erreicht werden[6]. In diesem Modell wird das Spenderherz (zusätzlich zum Empfängerherz) an den Kreislauf des Empfängers angeschlossen und perfundiert, übernimmt jedoch keine lebenserhaltende Funktion. Als Grundlage für einen klinischen Einsatz beim Menschen muss jedoch ein Langzeitüberleben nach vollständigem Herzersatz (orthotopes Transplantationsmodel) gelingen.

Gegenstand der vorliegenden Arbeit ist der Übertrag der Langzeiterfolge des heterotop-abdominellen Modells auf das lebenserhaltende orthotope Transplantationsmodell.

2. Strategien zur Überwindung von perioperativer Organdysfunktion

2.1 Perioperative Organdysfunktion nach Xenotransplantation

Organtransplantate müssen vom Zeitpunkt der Entnahme vom Spender bis zum Ende der Transplantation in den Empfänger präserviert werden, damit ihre Funktion erhalten bleibt. Klinischer Standard bei der Herztransplantation ist die Perfusion der Koronararterien mit einer kaliumreichen Lösung, die zur Asystolie führt (Kardioplegie); danach wird das Herz auf Eis gekühlt zum Empfänger gebracht. Durch Kardioplegie und Kühlung wird der Stoffwechsel des Herzens stark reduziert und die Ischämietoleranz auf 4-6 Stunden verlängert[7].

Trotz dieser Präservationsmaßnahmen kommt es nach Herztransplantation von Mensch zu Mensch in 4-7% der Fälle innerhalb der ersten 30 Tage zu einem Funktionsverlust des transplantierten Organs, was eine hohe Mortalität nach sich zieht[8,9]. Im Rahmen der orthotopen Xenotransplantion von genetisch modifizierten Schweineherzen auf Paviane ist ein ähnliches Phänomen beschrieben worden, die sogenannte „perioperative kardiale Xenograft-Dysfunktion“ (PCXD)[10]: Verschiedene Arbeitsgruppen hatten in den ersten 48 Stunden nach Operation von einem Funktionsverlust bis hin zum Versagen des porzinen Transplantats in 40-60% der Experimente berichtet. In keinem dieser Fälle konnten Hinweise auf eine hyperakute Abstoßung gefunden werden. Neben präformierten non-Gal-Antikörper gegen speziesfremde Epitope des Schweines und dadurch bedingte Aktivierung des teilweise inkompatiblen Gerinnungssystems des Primaten wurden für diese Reaktion insbesondere Ischämie-/Reperfusionsschäden verantwortlich gemacht. Interessanterweise ist auch nach kardialer Allotransplantation von Schwein zu Schwein von einer hohen Komplikationsrate und Transplantatdysfunktion von mehr als 50% berichtet worden[11,12]. Dies wirft die Frage auf, ob PCXD ein allgemeines Problem bei der Transplantation von ischämisch präservierten Schweinherzen darstellt und nicht spezifisch für die Xenotransplantation ist.

Voraussetzung für konsistente Langzeitergebnisse nach kardialer Xenotransplantation ist die Beherrschung der hohen Rate an postoperativer Organdysfunktion, was im Tierversuch mit dem Versuchsabbruch gleichzusetzen ist.

2.2 Vorarbeiten der Arbeitsgruppe: Ischämische Präservation der Schweineherzen

Aufbauend auf den Arbeiten von Mohiuddin et al. führte unsere Arbeitsgruppe orthotope Xenotransplantationen von Schweineherzen in Paviane durch. Die für diese Versuche eingesetzten genetischen Modifikationen der Spenderschweine (αGAL-KO/hCD46/hTBM) und das postoperative Immunsuppressionsregime - Kostimulationsblockade (CD40/CD40L) in Kombination mit Mykophenolat-Mofetil und Steroiden - hatte im heterotop-abdominellen Modell ein Überleben von mehr als 900 Tagen ermöglicht[6].

In einer ersten Gruppe (n=5) wurden die Spenderorgane nach klinischem Standard mit kristalloiden Lösungen kardioplegiert, entweder mit University of Wisconsin (UW; n=2) oder Histidin-Tryptophan-Ketogluterat (HTK; n=3) Lösung. Die Präservation bis zur Implantation erfolgte statisch-ischämisch bei 4°C. Trotz kurzer Ischämiezeiten (123±7 min) zeigte sich postoperativ eine deutliche Reduktion der Herzauswurfleistung (Abb. 1a) im Vergleich zum Eigenherz des Empfängers bei gleichzeitig hohem Bedarf an vasokonstriktiv (Abb. 1b) und inotrop (Abb. 1c) wirkenden Katecholaminen. Als Hinweis auf eine zunehmende Kreislaufinsuffizienz mit peripherer Minderversorgung des Gewebes und vermehrter Sauerstoffausschöpfung fiel die zentralvenöse Sättigung (Abb 1d), während die Laktatspiegel im Verlauf stetig stiegen (Abb. 1e). In drei von fünf Versuchen mussten die Experimente innerhalb der ersten 24h bei Multiorganversagen beendet werden; ein viertes Tier wurde nach drei Tagen aufgrund von Nierenversagen euthanasiert. Nur ein Tier brachte die perioperative Phase erfolgreich hinter sich und überlebte 30 Tage. Diese enttäuschend hohe Rate an PCXD von 60% war vergleichbar mit den Angaben in der Literatur[10].

Gegenüber HTK-Lösung hatte die ischämische Präservation mit UW-Lösung einen signifikanten Überlebensvorteil (p=0.0455). Von den fünf Tieren hatten diejenigen, welche UW-präservierte Herzen erhalten hatten, postoperativ die höchsten zentralvenösen Sättigungen, die geringsten Laktatwerte und längere Überlebenszeiten (3 und 30 Tage). Ein derartiger Unterschied zwischen UW- und HTK-Lösung ist beim Menschen nicht beschrieben[7].

Die Ergebnisse dieser ersten Gruppe machen deutlich, dass Perfusion mit klinischer „Standard"-Kardioplegie-Lösung und anschließender ischämischer Präservation keine adäquate Methode ist, um reproduzierbare Langzeitergebnisse nach Xenotransplantation zu erzielen.

2.3 Kontinuierliche nicht-ischämische Perfusion zur Präservation

In einer zweiten Gruppe (n=4) und allen darauffolgenden Versuchen wandten wir eine experimentelle Methode zur nicht-ischämischen Organpräservation an (in Kooperation mit S. Steen, Universität Lund, Schweden).

Steen et al. konnten mit dieser Präservationsmethode erfolgreiche orthotope Herztransplantation von Schwein zu Schwein nach einer Präservationszeit von 24 Stunden durchführen, wobei die myokardiale Kontraktilität vollständig erhalten blieb[13,14]. Als Präservationslösung dient hierbei eine 8°C kalte,

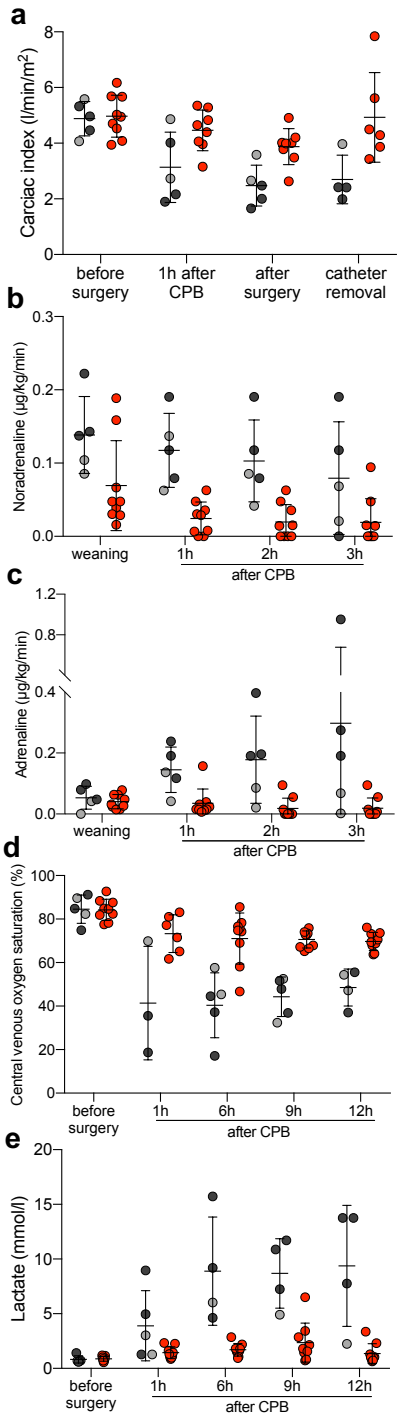

Abb. 1: Messungen von Kreislauffunktionen vor und nach orthotoper kardialer Xenotransplantation mittels Herzlungenmaschine (CPB) zu verschiedenen Zeitpunkten und Katecholaminbedarf. Die porzinen Spenderherzen wurden vor Transplantation in die Empfängerpaviane entweder kontinuierlich nicht ischämisch mit Präservationslösung perfundiert (rot, n=9) oder mittels kristalloider Lösungen kardioplegiert (schwarz: HTK (n=3); grau: UW (n=2)) und ischämisch präserviert. Abbildungen aus [15]

a) Herzindex (HI). Tiere mit perfundierten Herzen zeigten nach Transplantation einen erhaltenen HI. Im Gegensatz dazu war der HI bei Tieren mit ischämisch präservierten Herzen stark reduziert und erholte sich im operativen Verlauf nicht (p=0.0036).

b) Noradrenalin. Nach Transplantation von ischämisch präservierten Herzen waren deutlich höhere Dosen an Vasopressoren zur Kreislaufstabilisierung nötig als bei perfundierten Herzen (p=0.0021).

c) Adrenalin. Tieren mit ischämisch präservierten Herzen hatten einen signifikant höheren Bedarf an inotroper Unterstützung (p=0.0167); dieser stieg während der Operation zudem stetig an, während er bei
Tieren mit perfundierten Herzen abnahm (p=0.0122).

d) zentralvenöse Sättigung (SpO_2cv). Die SpO_2cv war bei Tieren mit ischämisch präservierten Herzen stark erniedrigt (p<0.0001) und blieb innerhalb der ersten 12h nach Herzlungenmaschine im pathologischen Bereich.

e) Serumlaktat. Während sich die Laktatwerte in Tieren mit perfundierten Herzen nach 12h normalisiert hatten, stiegen die Werte in Tieren mit ischämisch präservierten Herzen auf hoch pathologische Werte an (p<0.0001). Zusammen mit der SpO_2cv spricht dies für eine ausgeprägte Gewebsminderperfusion und periphere Hypoxie.

oxygenierte, albuminhaltige, hyperonkote, kardioplege Nährlösung, die mit Hormonen (Katecholaminen, Schildrüsenhormonen, Insulin) und Erythrozyten angereichert ist (Abb. 2a). Mittels der Präservationslösung wird das Spenderherz kardioplegiert und nach Explantation über eine temporäre Kanüle in der Aortenwurzel in einem extrakorporalen Herz-Präservations-System kontinuierlich perfundiert; nur während der Implantation erfolgt die Perfusion diskontinuierlich alle 15 min für jeweils 2 min (Abb. 2b). Das transportable Herz-Präservations-System besteht aus druck- und flusskontrollierten Rollerpumpen, einem O_2/CO_2-Austauscher, einem Leukozytenfilter und einem Heiz-/Kühlaggregat; das Reservoir mit kalter Präservationslösung dient zugleich als Aufbewahrungsort für das Organ.

a

Na^+	136 mmol/L
K^+	23 mmol/L
Ca^{2+}	1.3 mmol/L
Mg^{2+}	8.0 mmol/L
Cl^-	142 mmol/L
HCO_3^-	25 mmol/ml
PO_4^{2-}	1.3 mmol/L
D-Glucose	6.3 mmol/L
Albumin	75 g/L
Cocaine	6 nmol/L
Noradrenaline	6 nmol/L
Adrenaline	6 nmol/L
T3	3 nmol/L
T4	2 nmol/L
Cortisol	420 nmol/L
Insulin	8 U/L
Imipenem	20 mg/L
Erythrocytes (Hct)	10-15%
96% O_2 + 5 % CO_2	0.2 L/min

b

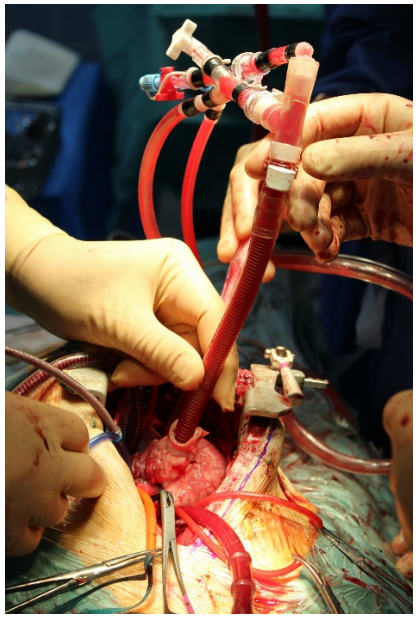

Abb. 2: **a)** Zusammensetzung der Präservationslösung für die kontinuierliche, nicht ischämischen Perfusion der Spenderorgane (modifiziert nach Steen et al.[13]). **b)** diskontinuierliche Perfusion des Herzens während der Implantation. Mittig abgebildet ist die temporäre Kanüle in der Aortenwurzel, über welche die Perfusionslösung bei geschlossener Aortenklappe in die Koronararterien gepumpt wird.

In unserer Versuchsreihe zeigten alle so behandelten Schweineherzen trotz längerer Präservationszeiten (206±43 min) nach Implantation in den Primaten eine gute Organfunktion. Die Herzzeitvolumina der Xenotransplantate waren postoperativ ähnlich hoch wie präoperativ (Abb. 1a); zugleich wurden signifikant geringere Dosen von kreislaufunterstützenden Medikamenten zur Stabilisierung der Tiere gebraucht (Abb. 1b,c). Im Gegensatz zur ischämischen Präservation fanden sich keine Hinweise auf Kreislaufinsuffizienz: die zentralvenöse Sauerstoffausschöpfung war nicht relevant erhöht und die Laktatwerte stiegen im postoperativen Verlauf nur verhältnismäßig leicht an (Abb.

1d,e). Alle Tiere überstanden die ersten 48 Stunden ohne Organschäden; ein Tier musste am vierten Tag wegen technischen Versagens euthanasiert werden, die anderen drei lebten 18, 27 und 40 Tage.

Wie von Steen at al. für die allogene Transplantation von Schweineherzen beschrieben minimierte die kontinuierliche, nicht-ischämischen Perfusion auch in unserem Modell die myokardiale Ischämie des Spenderorgan durch Beimischung von oxygenierten Erythrozyten zur Perfusionslösung, Senkung des Metabolismus durch Hypothermie und Kardioplegie und Aufrechterhaltung der nutritiven Versorgung und Abtransport von toxischen Metaboliten[13]. Zudem verhinderte der hohe onkotische Druck und die strenge Druck- und Flusskontrolle der Präservationslösung die Ödembildung durch Reduktion des Kapillarschadens; die Aufrechterhaltung von physiologischen Konzentrationen von Katecholaminen und Schilddrüsenhormonen gewährleisteten eine adäquate Pumpfunktion nach Präservation[14].

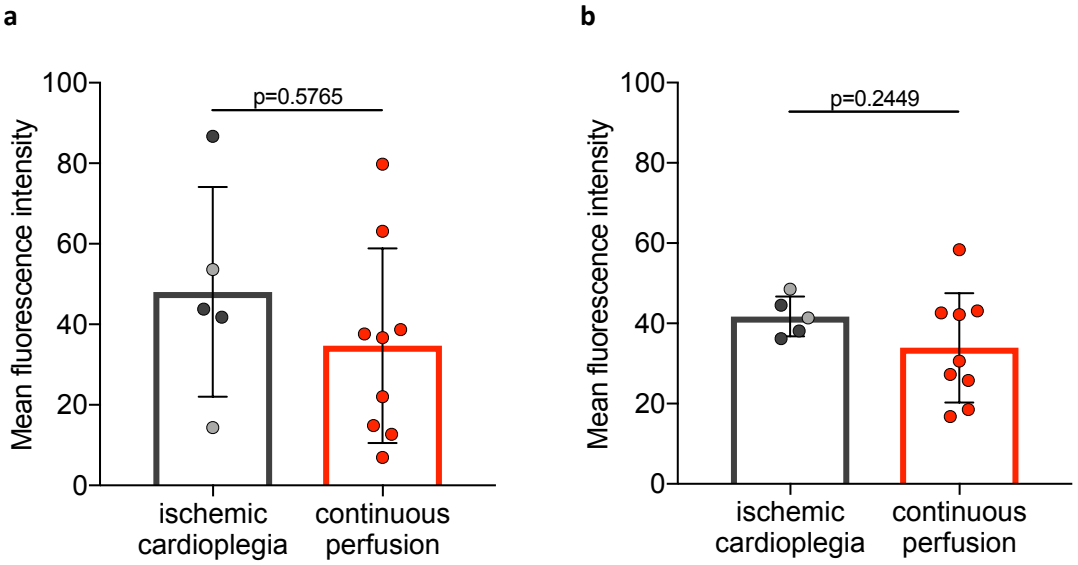

Abb. 3: Präoperative Serum-Titer von präformierten Anti-Non-Gal-Antikörpern (**a**, IgM; **b**, IgG). Zwischen den beiden Präservationsmethoden gab es keine signifikanten Unterschiede bezüglich der Antikörpertiter der Empfängertiere. Abbildungen aus [15]

In den Versuchsgruppen der ischämischen und nicht-ischämischen Präservation unterschieden sich weder die präoperativen Anti-Non-Gal-Antikörper-Titer (Abb. 3a,b), noch die genetischen Modifikationen der Spender (GTTA1-KO/hCD46/hTBM). Der Einfluss von präformierten Anti-Non-Gal-Antikörpern und Inkompatibilitäten der Gerinnungssysteme zwischen Schwein und Pavian auf das perioperative Graftversagen erscheint deshalb gering. Vielmehr ist anzunehmen, dass der Ischämie-/Reperfusionsschaden selbst die hauptsächliche Ursache für das frühe systolische Pumpversagen der Herzen ist: Die postoperativen Troponinwerte als Surrogatparameter für den myokardialen Zelluntergang waren nach ischämischer Präservation achtmal höher als in der Perfusionsgruppe. Ausschlaggebend für diese - anders als beim Menschen - ausgeprägten negativen Auswirkungen der

ischämischen Präservation könnte dabei das Gefäßsystem des Empfängertiers sein (siehe auch Kapitel 3): Im Vergleich zum juvenilen Schwein ist der systemische Widerstand des erwachsenen Pavians deutlich höher. Ein durch Ischämie/Reperfusion bereits geschädigtes Schweineherz mit reduzierter Pumpfunktion kann bei hoher Nachlast kein ausreichendes Herzzeitvolumen generieren - ähnlich der Rechtsherzdekompensation nach Transplantation bei Patienten mit bestehender pulmonaler Hypertonie. Durch nicht-ischämische Präservation wird der Ischämie-/Reperfusionsschaden nahezu vollständig verhindert, wodurch optimale Bedingungen für die Funktion des Herzens im vaskulären Hochdrucksystems des Pavians gegeben sind.

Mit diesen Ergebnissen konnten wir zeigen, dass eine Organpräservation mit kontinuierlicher, nicht-ischämischer Perfusion das Auftreten von perioperativer kardialer Xenograft-Dysfunktion vollständig verhindern kann. Dies ist eine wichtige Voraussetzung für die erfolgreiche Durchführung von konsistenten präklinischen Xenotransplantationsexperimenten.

3. Strategien zur Hemmung des Organwachstums nach Transplantation

3.1 Überschießendes Transplantatwachstum und Herzversagen

Bei allen länger andauernden Versuchen der Gruppen I und II zeigten die transplantierten Schweineherzen - unabhängig von der intraoperativen Präservationsmethode - Zeichen von überschießendem Wachstum. Bei der Sektion der Tiere fiel eine ausgeprägte Verdickung vor allem der linken Ventrikel- und Papillarmuskulatur im Sinne einer konzentrischen Hypertrophie auf (Abb. 4).

a **b**

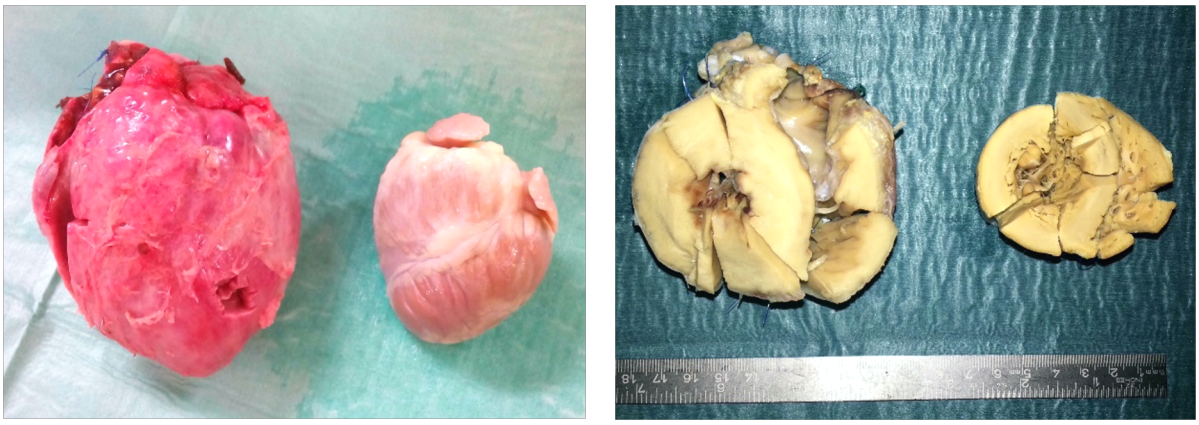

Abb. 4: Sektionspräparate (**a**) und in Formalin fixierte Querschnitte (**b**) des transplantierten Schweineherzens bei Versuchsende (jeweils links) nach 30 Tagen (Gruppe I) und des Pavianherzens (jeweils rechts). Am Tag der Transplantation hatten beide Herzen annähernd gleiche Größen. Auffällig sind die strak hypertrophierten Ventrikelwände und das beinahe verstrichene linksventrikuläre Lumen. Das Schlagvolumen dieses Herzen war zuletzt stark vermindert. Abbildungen aus [16]

a **b**

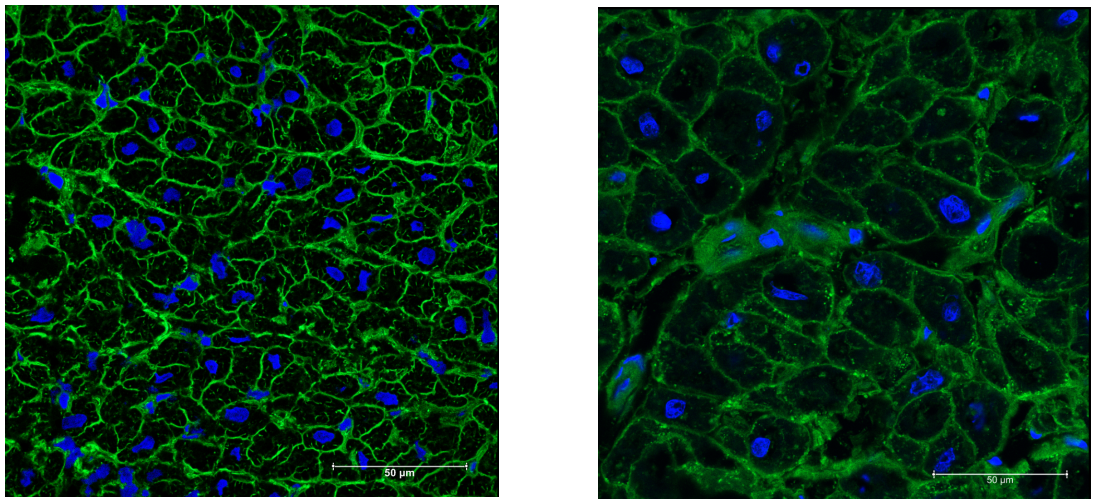

Abb. 5: Immunfluoreszenzanalysen (WGA-Färbungen) des Myokards bei gleicher Vergrößerung. **a**, normale Myokardzellen eines nicht transplantierten Schweineherzens. B, Myokardzellen eines Schweineherzen der Gruppe II 40 Tage nach Xenotransplantation in einen Pavian. Die mittlere Myokardfläche hat deutlich zugenommen (Kooperation mit R. Hinkel, TUM). Abbildungen aus [16]

In der histologischen Aufarbeitung konnten wir eine Zunahme der Kardiomyozytenfläche beobachten (Abb. 5). Die Hypertrophie der Ventrikelmuskulatur führte zur Störung der diastolischen Relaxation, zur Einengung der linken Herzkammer und zur Verminderung des Schlagvolumens. Laborchemisch zeigte sich im klinischen Verlauf dieser Tiere zunächst eine primäre Stabilisierung innerhalb der ersten beiden postoperativen Wochen; anschließend wies ein sekundär steigendes Troponin T auf einen zunehmenden Myokardschaden hin, während eine abnehmende Syntheseleistung (fallende CHE und Quick) und beginnende Organschäden (steigende ALT, AST und Bilirubin) der Leber als Zeichen von kardial bedingter Stauung auftraten. In keinem dieser Versuche wurden histologische bzw. serologische Hinweise auf eine humorale Abstoßung gefunden (Abb. 6).

a **b**

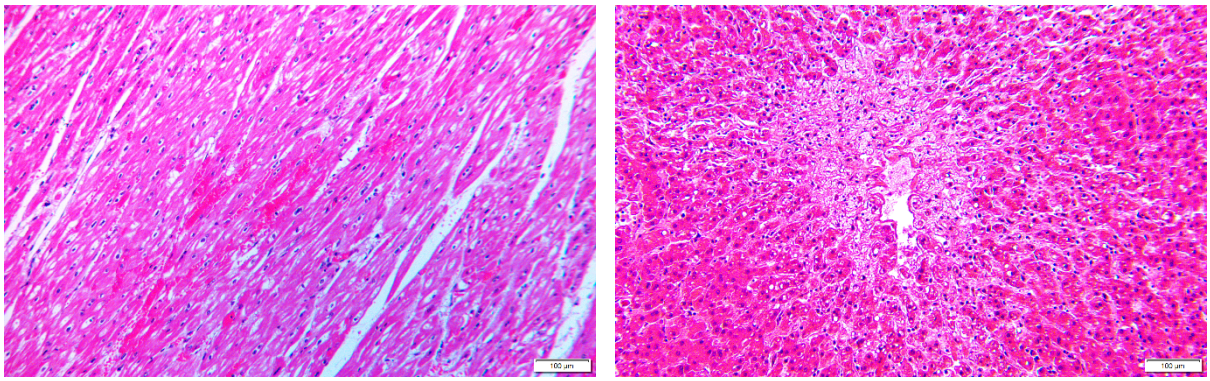

Abb. 6: HE-Färbungen von Myokard des Spenderorgans (**a**) und Lebergewebe des Empfängers (**b**) bei Versuchsende nach 40 Tagen (Gruppe II). Im Myokard waren Zellnekrosen, hämorrhagische Regionen und thrombotische Verschlüsse von kleinen Gefäßen zu erkennen, jedoch fanden sich keine Hinweise auf eine zelluläre oder humorale Abstoßung. In der Leber zeigten sich multiple zentrolobuläre Zellnekrosen und Blutungen. Diese Veränderungen sind vereinbar mit einem diastolischen Pumpversagen und dadurch bedingter akuter Leberstauung. Abbildungen aus [16]

3.2 Ursachen für Transplantatwachstum

Die Ursachen für das Wachstum von Spenderorganen nach Xenotransplantationen sind noch nicht eindeutig geklärt, obwohl Wachstum bereits in unterschiedlichen Modellen von verschiedenen Arbeitsgruppen beobachtet wurde[6,17,18]. Mögliche Erklärungsansätze, denen wir im Rahmen der vorliegenden Arbeit nachgingen, sind unter anderem i) physiologisches Wachstum auf Grund eines Größen-Missmatches, ii) pathologische Hypertrophie auf Grund von Inkompatibilitäten zwischen den Tiermodellen, wie z.B. Hämodynamik und iii) Nebenwirkungen der immunsuppressiven oder supportiven Behandlung. Im Folgenden sollen diese drei Punkte näher betrachtet werden.

i) Im lebenserhaltenden orthotopen Xenotransplantationsmodel dienen adoleszente Paviane im Alter von 3-6 Jahren als Organempfänger. Üblicherweise haben diese Tiere ein Körpergewicht zwischen 15

und 25 kg, abhängig von Alter, Ernährung und Rang in der Gruppe. In dieser Altersgruppe nimmt das Gewicht der Tiere nur noch langsam zu (wenige kg/Jahr). Ähnlich wie bei Allotransplantationen beim Menschen werden auch im Xenotransplantationsmodell Empfänger und Spender nach Körpergewicht „gematched", damit die Organe ähnliche Größen haben. Sowohl „oversizing" (zu großes Organ) als auch „undersizing" (zu kleines Organ) können perioperativ Probleme bereiten. Im ersteren Fall ist nicht genug Platz im Thorax, im letzteren Fall kann das neue Organ auf Grund seiner zu kleinen Größe nicht ausreichend Schlagvolumen generieren, um einen suffizienten Kreislauf zu sichern. Aus retrospektiven Daten unserer Versuchstiere wissen wir, dass das Herzgewicht des Pavian 0.43±0.06% des Körpergewichts beträgt, während das Schweineherz bei gleichem Körpergewicht etwa 50% schwerer ist (0.66±0.10%). Dieser Unterschied kann durch Wahl eines kleineren Spenders ausgeglichen werden. Ein viel gravierenderer Unterschied zwischen den Spezies Pavian und Hausschwein ist jedoch das viel schnellere Organwachstum und das erreichbare Endgewicht. Bei gleichem Gewicht (15-25 kg) ist das Schwein zum Zeitpunkt der Transplantation nur wenige Wochen alt und an der steilsten Stelle seiner Wachstumskurve. Innerhalb der folgenden 3 Monate würde sich sein Gewicht vervierfachen; die Größenzunahme der Organe folgt ungefähr der Zunahme des Körpergewichts. Bei abdominellen Nierentransplantationen von Hausschweinen auf kleinere Minipigs konnte dieser Effekt nachgewiesen werden: die Nieren der Hausschweine zeigten in den langsamer wachsenden Minipigs die „normale" Wachstumsgeschwindigkeit des Spenders[18]. Am Ende der Versuche nahmen die Spendernieren den Großteil des Bauchraumes der Versuchstiere ein. Im heterotop abdominellen kardialen Xenotransplantationsmodell wuchsen die Spenderherzen zwar ebenfalls, erreichten jedoch nur das Gewicht der Pavianeigenherzen und nicht das um ein Vielfaches höhere Endgewicht nicht transplantierter Kontrollschweine[6]. Es ist also naheliegend, dass im orthotopen Xenotransplantationsmodel noch weitere Mechanismen das Organwachstum beeinflussen.

ii) Das Schwein wird nicht zuletzt wegen der Ähnlichkeiten des kardiovaskulären Systems als idealer Organspender für den Menschen angesehen[19]. Der im lebenserhaltenden orthotopen Xenotransplantationsmodel eingesetzte Pavian unterscheidet sich jedoch vom Schwein: während die beiden Spezies ähnliche Herzindizes und Herzfrequenzen aufweisen, sind Pavianherzen eine deutliche höhere Nachlast gewöhnt. Wie wir zeigen konnten, haben adoleszente Paviane einen um 60% höheren arteriellen Mitteldruck und einen um 121% höheren systemvaskulären Widerstandsindex als junge Schweine mit vergleichbarem Gewicht (Abb. 7).

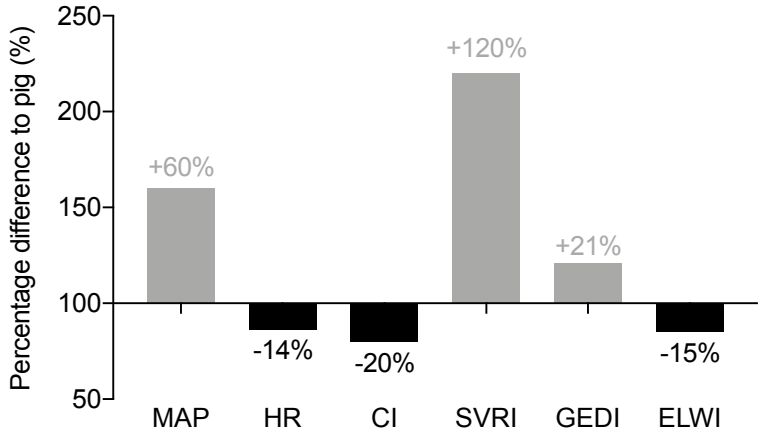

Abb. 7: Vergleich hämodynamischer Parameter von narkotisierten ausgewachsenen Pavianen und juvenilen Schweinen. Während sich Herzfrequenz (HR) und Herzindex (CI) nur geringfügig unterscheiden, fallen vor allem die höheren Werte von mittlerem arteriellem Blutdruck (MAP) und systemvaskulären Widerstandsindex (SVRI) beim Pavian auf. Abbildung aus [20]

Mit zunehmenden Alter der Schweine nehmen auch Blutdruck und systemischer Widerstand zu, um im ausgewachsen Zustand menschliche Normwerte zu erreichen oder sogar zu übertreffen[21]. Bei Menschen mit essentieller Hypertonie führt ein chronisch erhöhter systemvaskulärer Widerstand zu pathologischer Myokardhypertrophie und – bei Ausschöpfung der Kompensationsmechanismen – zu kardialem Pumpversagen[22]. Nach Transplantation des an niedrige Systemwiderstände gewöhnte junge Schweineherz in den Pavianen mit mehr als doppelt so hohem Widerstand wird möglicherweise eine chronische Adaptation ausgelöst, die schlussendlich im diastolischen Pumpversagen mündet. Im heterotop abdominellen Modell wurde keine pathologische Hypertrophie beobachtet[6], während im heterotop thorakalen Modell die Hypertrophie durch Verdrängung der Lungen versuchslimitierend war[17]. Im ersteren, nicht lebenserhaltenden Model wird das Xenograft nur perfundiert und muss nicht gegen den Systemwiderstand auswerfen; im letzteren, teilweise lebenserhaltenden Model trägt das Spenderherz zusammen mit dem Empfängerherz zum Herzzeitvolumen bei.

iii) Auch Nebenwirkungen von Medikamenten können für ein unphysiologisches Myokardwachstum verantwortlich sein. So kann die chronische Behandlung mit hohen Dosen von Kortikosteroiden – essentieller Bestandteil des begleitenden immunsuppressiven Therapieschemas – bei Neugeborenen und Kindern eine Myokardhypertrophie auslösen[23]. In der postoperativen Behandlung nach Kinderherztransplantation ist dieses Problem bekannt, weshalb dort die Reduktion von Kortikosteroiden sehr rasch erfolgt.

Sowohl natürliches Wachstum als auch pathologische Hypertrophie sind mögliche Ursachen für eine überschießende Größenzunahme des Xenografts, was schließlich in Herz- und Leberversagen der Versuchstiere mündet. Eine Hemmung dieses Wachstums ist deshalb Grundvoraussetzung, um eine Versuchsdauer von 3 Monaten zu erreichen.

3.3 Therapieregime zur Hemmung des Transplantatwachstums

Aufbauend auf den Erkenntnissen aus den ersten beiden Gruppen entwickelten wir ein Therapieregime mit dem Ziel, überschießendes Wachstum des Xenografts zu hemmen. Dieses Regime beinhaltete drei Bestandteile:

i) Rasche Kortisonreduktion: Die immunsuppressive Kortisontherapie, beginnend mit 10mg/kg/d am Operationstag, wurde innerhalb von 19 Tagen auf die Erhaltungsdosis von 0.1mg/kg/d reduziert. Das bisherige Therapieschema sah eine Reduktion um 1mg/kg/d alle 7 Tage vor. Durch das schnelle Ausschleichen sollte die Entstehung einer kortisoninduzierten Myokardhypertrophie vorgebeugt werden.
ii) Antihypertensive Behandlung: Postoperativ wurde so bald als möglich eine Behandlung mit je einem beta-Blocker (Metoprolol) und ACE-Inhibitor (Enalapril) begonnen, um den im Vergleich zum Schwein höheren systemischen Blutdruck des Pavians auf niedrig normale Level zu senken. Es erfolgte eine individuelle Dosissteigerung bis zum Erreichen der Zielwerte (arterieller Mitteldruck 80-90 mmHg, Herzfrequenz 80-120/min).
iii) Behandlung mit mTOR-Inhibitor: Der mTOR-Signalweg stellt eine zentrale Schaltstelle im Zellmetabolismus dar und ist unter anderem für die Regulation von Zellwachstum, Proliferation und Apoptose mitverantwortlich[24]. Durch die zusätzliche Gabe von Temsirolimus, eines intravenösen Prodrugs des Immunsuppressivums Sirolimus (Rapamycin), sollten Wachstum und Proliferation auf zellulärer Ebene inhibiert werden. Verschiedene klinische Studien haben gezeigt, dass unter Behandlung mit mTOR-Inhibitoren eine myokardiale Hypertrophie verringert und die diastolische Pumpfunktion verbessert werden[25-27]. Die tägliche Behandlung wurde eine Woche nach Transplantation begonnen, die Dosis wurde individuell nach Zielspiegel (4-8ng/ml) titriert.

3.4 Verhinderung von Transplantatwachstum und Organversagen

In einer dritten Gruppe (n=5) von orthotopen Xenotransplantationsversuchen setzten wir zusätzlich zur nicht-ischämischen Präservation mittels kontinuierlicher Perfusion das oben genannte wachstumsinhibierende Therapieschema ein. Genetische Modifikationen der Spendertiere und postoperative immunsuppressive Behandlung waren identisch zu Gruppe I und II.

Der initiale postoperative Verlauf war ähnlich zu jenem von Gruppe II: Alle Tiere überstanden problemlos die perioperative Phase, die Transplantatfunktionen waren sehr gut und hämodynamische Funktionsindizes blieben unverändert bei niedrigem Katecholaminbedarf (Abb. 1). Fälle von PCXD wurden nicht beobachtet, und alle Tiere erreichten innerhalb der ersten vier Wochen eine stabile

Organfunktion. Ein Tier musste nach 51 Tagen bei therapieresistentem Chylothorax auf Grund eines Verschlusses des Ductus thoracicus euthanasiert werden; die anderen vier Tiere der Gruppe erreichten bei gutem klinischem Zustand das als Voraussetzung zum Einsatz am Menschen angestrebte 90 Tage-Überleben (Abb. 8). In der versuchsbegleitenden transthorakalen Echokardiographie konnten wir - im Unterschied zu den Versuchen von Gruppe I und II - keine relevante Zunahme der Herzmuskeldicke oder Zeichen von Herzinsuffizienz feststellen (Abb. 9). Laborparameter von Herz- und Leberfunktion stabilisierten sich wenige Tage nach Operation innerhalb oder nahe der angegeben Normalwerte (Abb. 10).

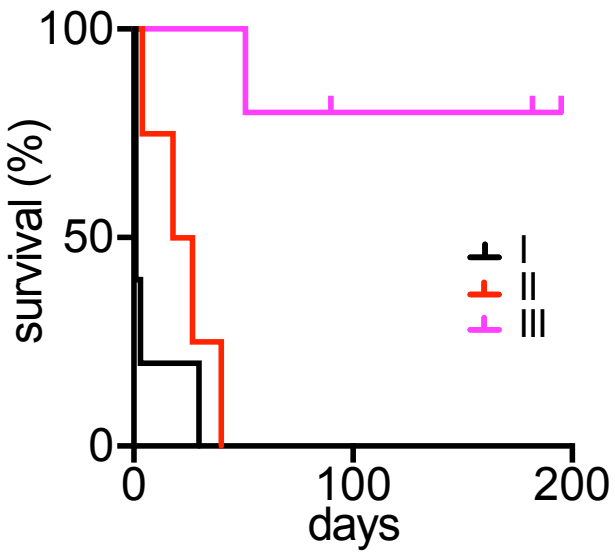

Abb. 8: Überleben von Pavianen nach orthotoper Xenotransplantation von genetisch modifizierten Schweineherzen. In Gruppe I (n=5, schwarz) mussten 60% der Versuche auf Grund von frühem Graftversagen (PCXD) beendet werden. In Gruppe II (n=4, rot) trat kein PCXD mehr auf, aber das überschießende Wachstum der Spenderherzen war versuchslimitierend. In Gruppe III (n=5, magenta) erreichten vier Tiere das Versuchsziel von 90 Tagen, zwei davon sogar 195 und 182 Tage. Alle vier Tiere wurden in gutem Allgemeinzustand euthanasiert. Abbildung (modifiziert) aus [16]

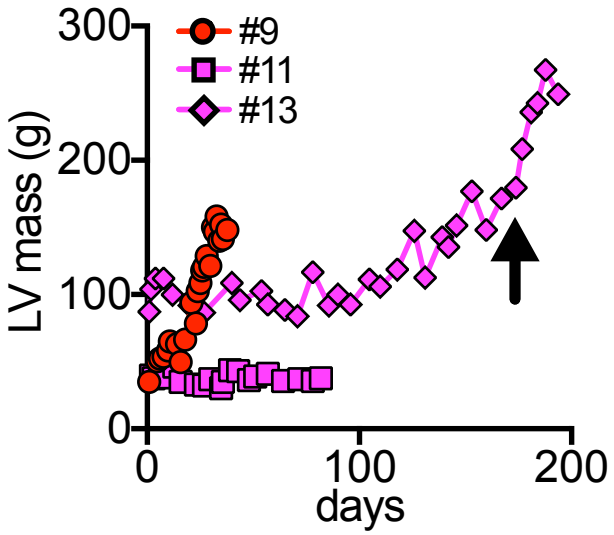

Abb. 9: Echokardiographisch bestimmte linksventrikuläre Masse von drei exemplarischen Versuchstieren. In Versuch #9 (Gruppe II) zeigte das Spenderherz ein ausgeprägtes Wachstum, das zum diastolischen Pumpversagen und zur Leberstauung führte. In Versuch #11 und #13 (Gruppe III) wurde das Transplantatwachstum medikamentös gehemmt. In den ersten 90 Tagen zeigte sich keine Zunahme der Herzmasse. Drei Wochen vor Ende des Versuchs #13 wurde die Behandlung mit Temsirolimus gestoppt (Pfeil); daraufhin folgte eine rasche Zunahme der linksventrikulären Masse. Abbildung (modifiziert) aus [16]

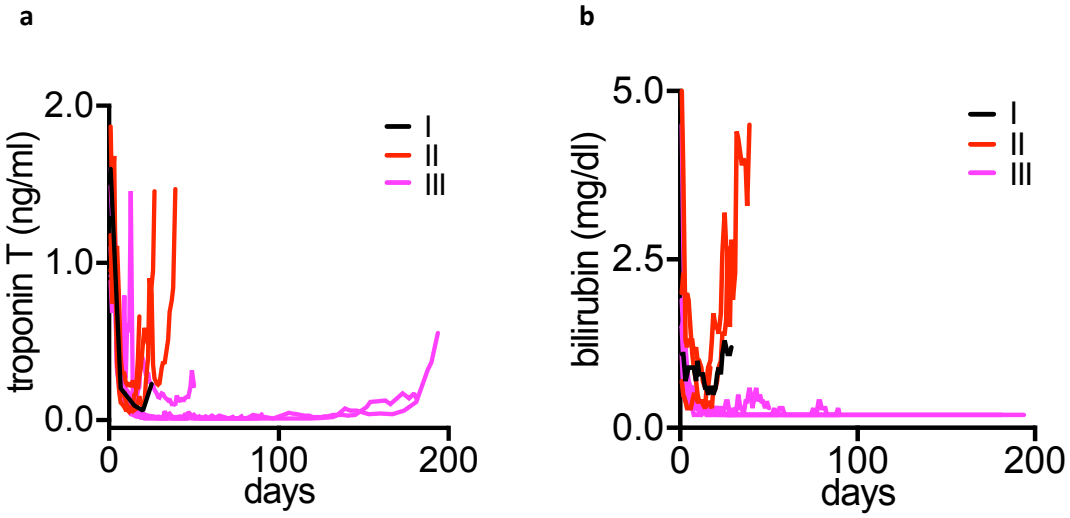

Abb. 10: Serumparameter der Herz- (**a**, Troponin T) und Leberfunktion (**b**, Bilirubin) der Paviane von Gruppe I-III. Während in Gruppe I und II nach initialer Stabilisierung ein Anstieg von Troponin T und Bilirubin das beginnende diastolische Pumpversagen und die konsekutive Leberstauung anzeigten, zeigten die Tiere der Gruppe III über den gesamten Versuchsverlauf annähernd normale Verlaufsparameter. Nach 6 Monaten stieg bei einem der Tiere nach Absetzen von Temsirolimus das Troponin rasch an. Abbildung (modifiziert) aus [16]

Nach 90 Tagen – und damit Erreichen des Studienziels - wurden zwei der Tiere gemäß Studienprotokoll euthanasiert. Bei Sektion der beiden Tiere fiel ein annähernd normales Myokard ohne Zeichen von

Hypertrophie auf (Abb. 11). In der histologischen Untersuchung hatten die Myokardzellen an Fläche nicht zugenommen (Abb. 12).

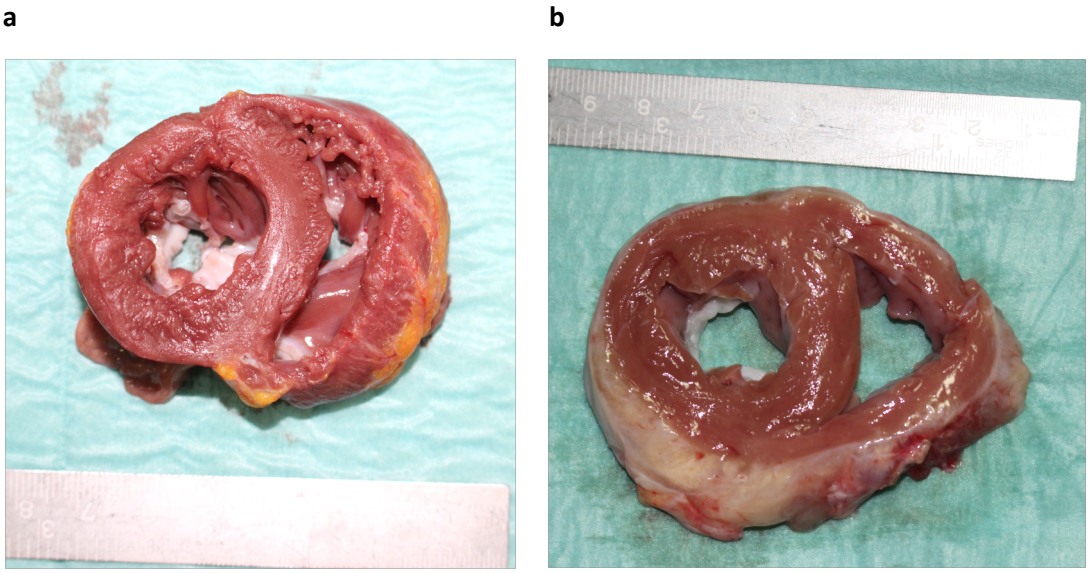

Abb. 11: Querschnitte knapp unterhalb der Klappenebene des Pavianeigenherzens am Tag der Transplantation (**a**) und des transplantierten Schweineherzens (Gruppe III) bei Versuchsende nach 90 Tagen (**b**). Im Gegensatz zu Herzen der Gruppen I und II (Abb. 5) hatte das Myokard nur wenig an Gewicht zugenommen, die Herzhöhlen waren normal weit. Abbildung (modifiziert) aus [16]

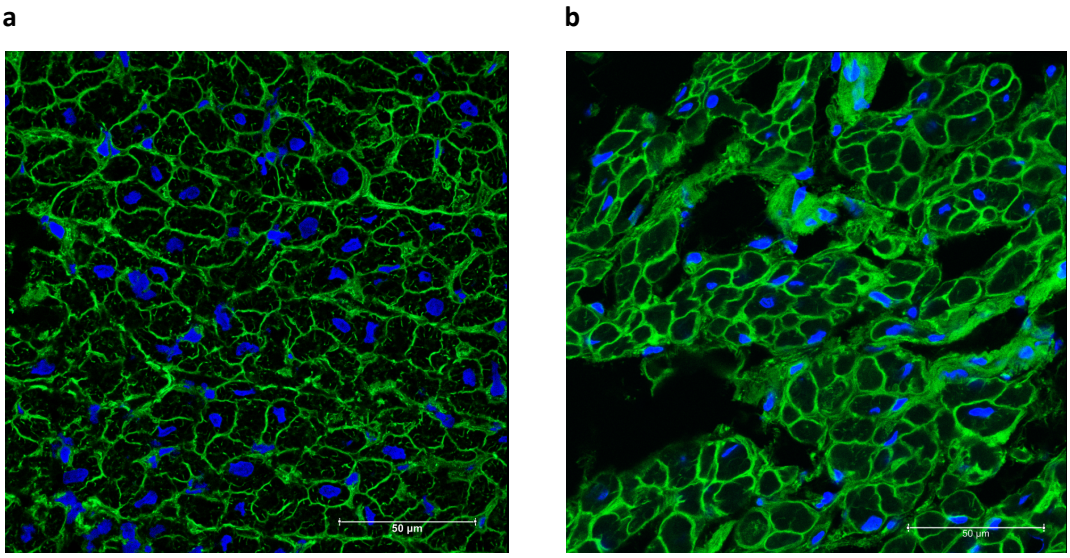

Abb. 12: Immunfluoreszenzanalysen (WGA-Färbungen) des Myokards in gleicher Vergrößerung. **a**, normale Myokardzellen eines nicht transplantierten Schweineherzens. **b**, Myokardzellen eines Schweineherzens der Gruppe III 90 Tage nach Xenotransplantation in einen Pavian. Die mittlere Myokardfläche hat nicht zugenommen. Abbildung aus [16]

Nach Genehmigung durch die Regierung führten wir die beiden anderen Versuche weiter und beendeten sie nach 195 und 182 Tagen (Abb. 8). Drei Wochen vor Versuchsende war die Behandlung mit Temsirolimus im Rahmen von Oralisierungsversuchen gestoppt worden. Nach Absetzen des mTOR-

Inhibitors nahm das Myokardwachstum bei einem der Versuchstiere (#13) in den echokardiographischen Untersuchungen rasch zu (Abb. 9); laborchemisch war ein Ansteigen der Herz- und Leberenzyme als Zeichen von beginnender Organdysfunktion aufgefallen (Abb. 10).
Auch in Gruppe III konnte in keinem der Versuche histologische bzw. serologische Hinweise auf eine relevante humorale oder zelluläre Abstoßung gefunden werden (Abb. 13).

a **b**

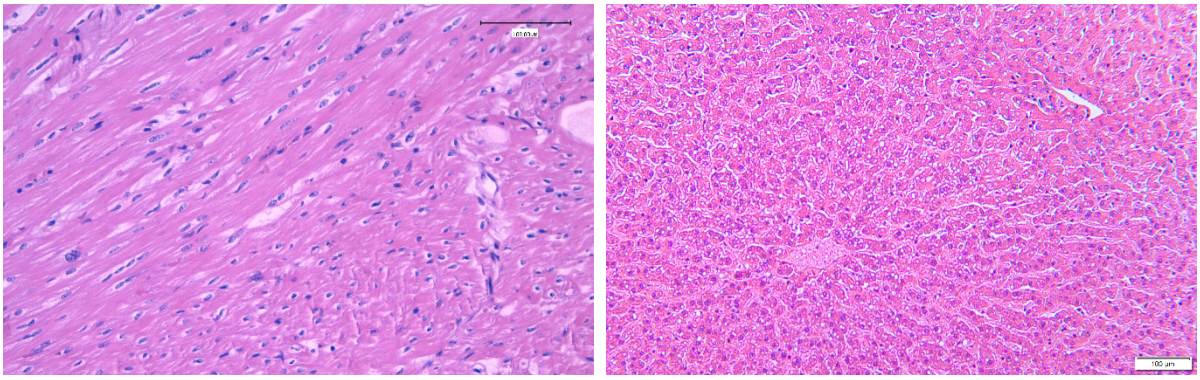

Abb. 13: HE-Färbungen von Myokard des Spenderorgans (**a**) und Lebergewebe des Empfängers (**b**) bei Versuchsende nach 90 Tagen (Gruppe III). Weder Myokard noch Lebergewebe zeigten signifikante pathologische Veränderungen, insbesondere keine Hinweise auf relevante zelluläre oder humorale Abstoßung. Abbildung aus [16]

Mit diesen Ergebnissen konnten wir zeigen, dass mit ausreichender Hemmung des Transplantatwachstums im Tierversuch ein Überleben nach kardialer Xenotransplantation für mehr als sechs Monate möglich ist. Der Stellenwert des mTOR-Inhibitors ist besonders herauszuheben; nach seinem Absetzen beginnt das Graft wieder zu wachsen.

4. Erfüllung der Voraussetzungen für erste klinische Xenotransplantationsversuche

Im Jahre 2000 hat das „Xenotransplantation Advisory Committee of the International Society of Heart and Lung Transplantation (ISHLT)“ Empfehlungen für experimentelle Xenotransplantationversuche veröffentlich, deren Erfüllung als Voraussetzung für den Beginn von klinischen Studien angesehen wird[28]. In Bezug auf die kardiale Xenotransplantation wurden die folgenden Kriterien formuliert: Ein Überleben des Transplantats - und damit des Empfängertieres - von mindestens 90 Tagen sollte in 60% einer Versuchsgruppe nachgewiesen werden. Als Transplantationsmodel wurde das lebenserhaltende orthotope Verfahren gerfordert, dabei sollten nicht-menschliche Primaten als Empfänger dienen.

Durch die Verbesserung der Präservationsmethoden und der medikamentösen Prävention von überschießendem Organwachstum erreichten wir in Gruppe III erstmals ein konsistentes Überleben von mindestens 90 Tagen. Mit dem Ziel alle Vorgaben der ISHLT zu erfüllen, wurde nur der Arm der Versuchsgruppe III fortgesetzt, der bisher die besten Ergebnisse erzielt hatte (Kostimulationsblockade mit chimären anti-CD40-Antikörper). Zusätzlich zu diesen vier erfolgreichen Versuchen (Überleben: 90d, 90d, 195d und 182d) wurden vier weitere Versuche durchgeführt. Die ersten beiden mussten auf Grund eines Multiorganversagens nach 15 und 27 Tagen beendet werden. Die Empfänger hatten Organe von Spendern bekommen, die positiv auf porzines Cytomegalie-Virus (pCMV) getestet wurden. pCMV wurde nach Xenotransplantation von Nieren mit deutlich vermindertem Transplantatüberleben in Verbindung gebracht[29], wobei der hierfür zugrunde liegende Mechanismus bisher nicht geklärt ist[30]. Akute zelluläre oder humorale Abstoßungen als Ursache für die frühen Versuchsabbrüche konnten ausgeschlossen werden (Abb. 14).

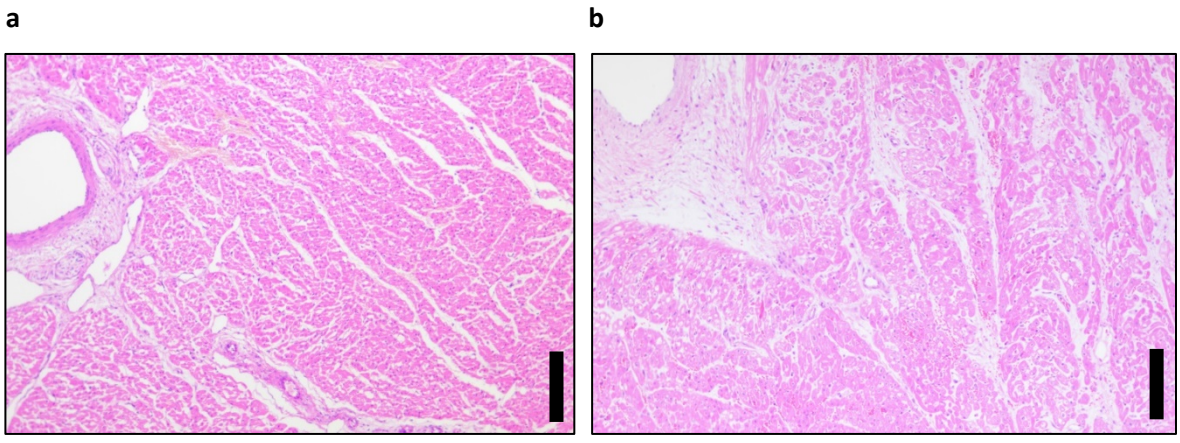

Abb. 14: HE-Färbungen von Myokard der beiden Spenderorgane, die positiv auf porzines Cytomegalie-Virus getestet wurden. Die Empfänger mussten nach 15 (**a**) und 27 Tagen (**b**) wegen Multiorganversagen euthanasiert werden. Die Histologie zeigt ein moderates perivaskuläres und interstitielles Ödem in sonst unauffälligem Gewebe, insbesondere keine morphologischen Hinweise auf akute zelluläre oder humorale Abstoßung (Maßstab 250µm). Abbildung (modifiziert) aus [31]

In den beiden folgenden Versuchen wurden nur noch Herzen von pCMV negativen Schweinen transplantiert, welche früh von den Muttersauen entwöhnt wurden, um eine postnatale Übertragung des Virus zu verhindern[32]. Diese konnten erfolgreich über den definierten Zeitraum von 90 Tagen durchgeführt werden; die Tiere wurden in gutem Allgemeinzustand euthanasiert. Sowohl die hämodynamischen Parameter, die Ergebnisse der echokardiographischen Untersuchungen, als auch der Verläufe der Laborparameter waren vergleichbar mit den Resultaten der anderen Tiere von Gruppe III. Makroskopisch waren auch bei diesen Herzen keine Zeichen einer überschießenden linksventrikulären Hypertrophie zu erkennen, die Ventrikellumina waren nicht reduziert (Abb. 15). Die Histologie war bis auf milde bis moderate perivaskuläre und interstitielle Ödeme unauffällig; bei einem Tier war fokal eine geringgradige akute zelluläre Reaktion zu erkennen, die klinisch unauffällig geblieben war (Abb. 16).

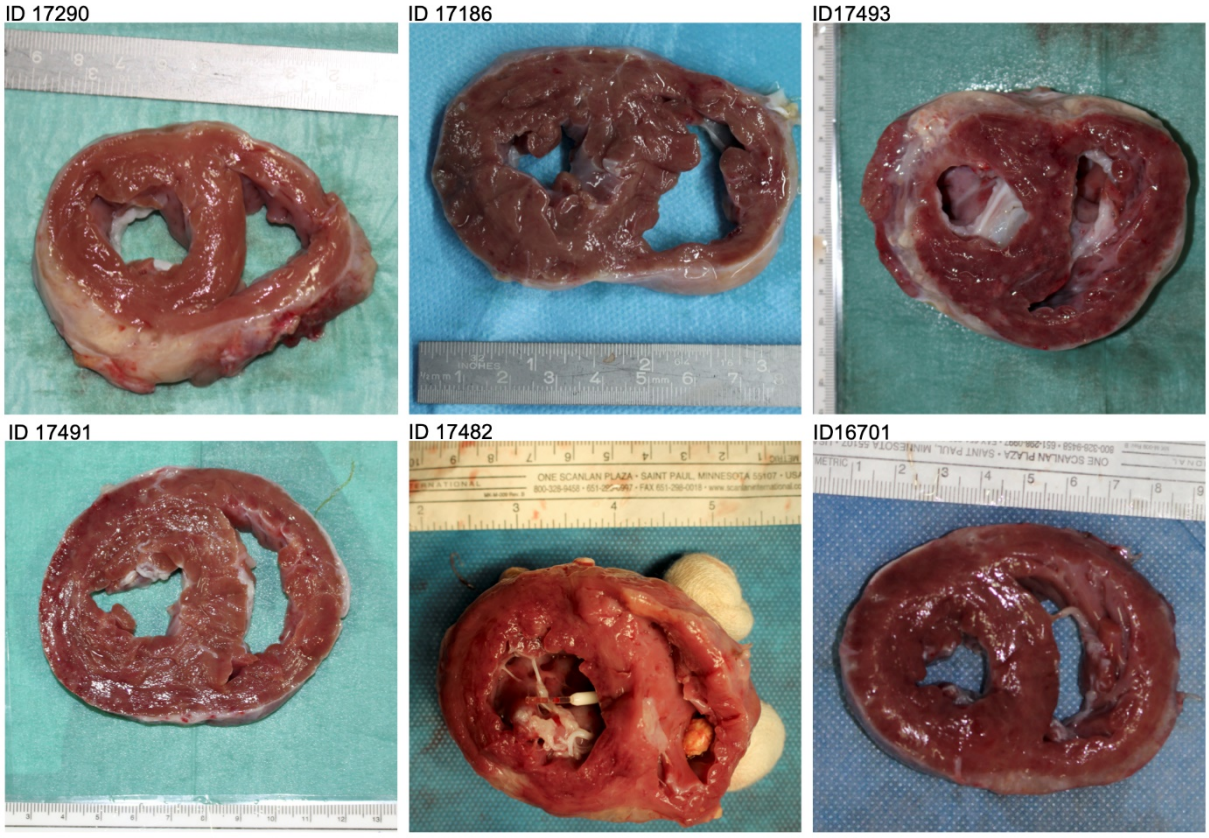

Abb. 15: Querschnitte knapp unterhalb der Klappenebene der transplantierten Schweineherzen die mindestens 90 Tage nach Xenotransplantation überlebten (ID17493: 195d; ID17491: 182d). Abbildung (modifiziert) aus [33]

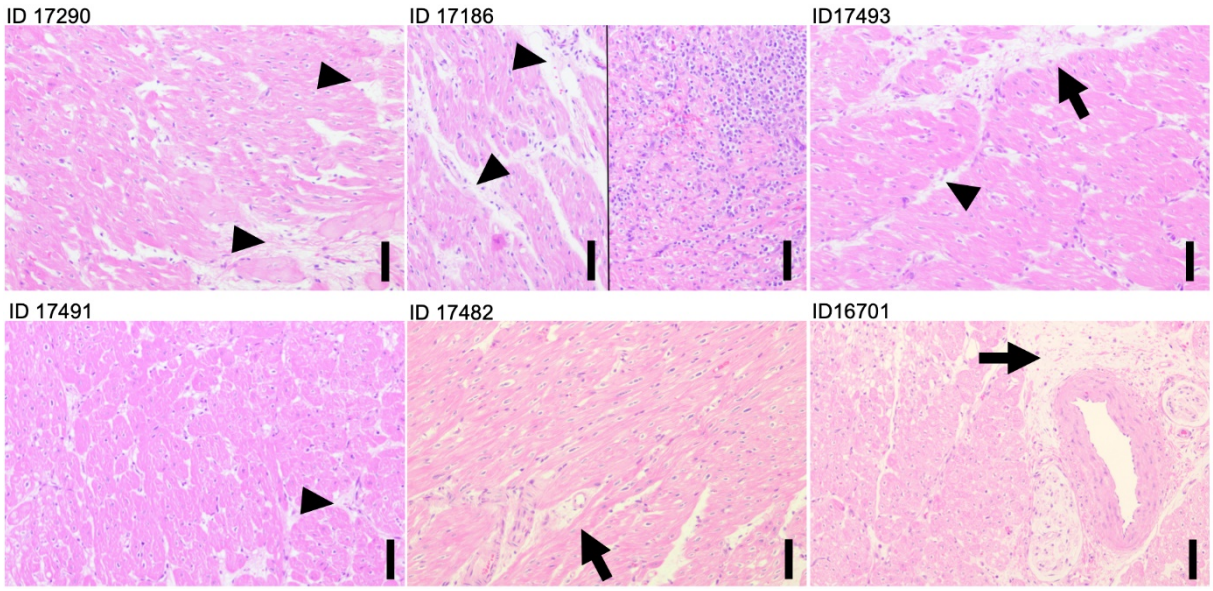

Abb. 16: HE-Färbungen von Myokardproben der Herzen aus Abb. 16. In allen Proben waren milde bis moderate perivaskuläre (Pfeil) und interstitielle (Pfeilspitze) Ödeme finden. Bei einem Tier (ID 17186, rechte Seite) zeigte sich ein solitäres fokales inflammatorisches Infiltrat, welches auf eine geringgradige zelluläre Reaktion schließen lässt (Maßstab 100µm). Abbildung aus [33]

Mit diesen Ergebnissen (sechs von acht konsekutiven Tieren einer Gruppe mit mindestens 90 Tagen Überleben nach lebenserhaltender orthotoper kardialer Xenotransplanation) konnten wir erstmals die von internationalen Experten empfohlenen Voraussetzungen für den klinischen Einsatz der kardialen Xenotransplantation erfüllen.

5. Zusammenfassung und Ausblick

Nach wie vor ist die allogene Herztransplantation der therapeutische Goldstandard für die terminale Herzinsuffizienz. Bei der weiter bestehenden Organknappheit scheint die kardiale Xenotransplantation eine vielversprechende Alternative zur allogenen Transplantation zu sein. Bislang konnten jedoch längere Überlebenszeiten der Spenderorgane nur im nicht lebenserhaltenden, heterotop abdominellen Xenotransplantationsmodel erreicht werden. Die Ergebnisse der vorliegenden Arbeit zeigen erstmals, dass ein konsistentes Überleben auch im orthotopen Model möglich ist.

Die erste wichtige Voraussetzung ist die kontinuierliche, kalte, nicht-ischämische Perfusion der Spenderherzen zur Präservation zwischen Explantation und Implantation in den Empfänger. Dadurch kann das Auftreten eines perioperativen systolischen Pumpversagens vollständig vermieden werden, was die Basis für konsistente präklinische und erste klinische Versuche darstellt.

Eine wichtige Voraussetzung ist auch die genetische Modifikation der Spendertiere mit mindestens αGAL-Knockout, Expression von humanem CD46 und Thrombomodulin zur Verhinderung von hyperakuten Abstoßungsreaktionen und zur Komplement- und Gerinnungsmodulation. Für den Einsatz beim Menschen werden voraussichtlich noch weitere genetische Modifikationen nötig sein, wie z.B. die Entfernung von Xenoantigenen, gegen die nicht-menschliche Primaten im Gegensatz zu Menschen keine präformierten Antikörper besitzen (z.B. N-Glycolylneuraminsäure) oder die Expression von zusätzlichen humanen Faktoren. Die „beste“ Kombination der möglichen genetischen Modifikation ist nach wie vor Gegenstand wissenschaftlicher Forschung.

Eine weitere Voraussetzung für ein Langzeitüberleben im orthotopen Transplantationmodell scheint - neben einer auf Koststimulation (anti-CD40-Antikörper) basierenden Immunsuppression - die medikamentöse Hemmung des überschießenden Transplantatwachstums und der dadurch bedingten Entwicklung eines chronischen diastolischen Pumpversagens zu sein: im Tiermodel konnte dies mittels einer Kombination aus rascher Steroidreduktion, antihypertensiver Medikation und antiproliferativer Behandlung mit einem mTOR-Inhibitor erreicht werden. In zukünftigen Studien muss untersucht werden, ob das überschießende Organwachstum eventuell auch durch genetische Modifikationen der Spendertiere (z.B. kleinwüchsige Tiere) verhindert werden kann.

Zu guter Letzt ist auch der Infektionsstatus der Spendertiere von großer Bedeutung: Organe von pCMV-positiven Tieren erreichen keine langen Überlebenszeiten, so dass in präklinischen, wie auch zukünftigen klinischen Studien ausschließlich Herzen transplantiert werden sollten, die von pCMV-negativen Spendertieren stammen.

Mit der Erfüllung der von internationalen Experten empfohlenen Voraussetzungen (90 Tage Überleben bei 60% der Tiere einer Versuchsgruppe nach orthotoper kardialer Xenotransplanation) erscheint nun der klinische Einsatz der kardialen Xenotransplantation greifbar nahe.

6. Danksagung

Der Arbeitsgruppe Xenotransplantation bin ich zutiefst dankbar für die Möglichkeit, meine Habilitationsarbeit anfertigen zu dürfen. Insbesondere danke ich Prof. Reichart, Prof. Brenner und PD Dr. Abicht für unzählige fruchtbare Diskussionen, viele schlaflose Nächte im OP und auf ICU und unvergleichliche Geschichten von den Anfängen der Xenotransplantationsforschung.

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7. Literatur

Eigene Publikation, die einen Teil dieser Habilitationsschrift darstellen, sind fettgedruckt, der eigene Name ist unterstrichen.

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8. Originalarbeiten

Auf den folgenden Seiten sind die dieser kumulativen Habilitationsschrift zugrunde liegenden Originalarbeiten dargestellt. Für den Gesamtkontext sei auf den Hauptteil der Habilitationsschrift verwiesen.

- **Längin M, Reichart B, Steen S, et al. Cold non-ischemic heart preservation with continuous perfusion prevents early graft failure in orthotopic pig-to-baboon xenotransplantation. Xenotransplantation. August 2020:e12636. doi:10.1111/xen.12636.**

- **Längin M, Mayr T, Reichart B, et al. Consistent success in life-supporting porcine cardiac xenotransplantation. Nature. 2018;564:430-433. doi:10.1038/s41586-018-0765-z.**

- **Längin M, Konrad M, Reichart B, et al. Hemodynamic evaluation of anesthetized baboons and piglets by transpulmonary thermodilution: Normal values and interspecies differences with respect to xenotransplantation. Xenotransplantation. 2019;65:565-569. doi:10.1111/xen.12576.**

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ORIGINAL ARTICLE

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Cold non-ischemic heart preservation with continuous perfusion prevents early graft failure in orthotopic pig-to-baboon xenotransplantation

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Abstract

Background: Successful preclinical transplantations of porcine hearts into baboon recipients are required before commencing clinical trials. Despite years of research, over half of the orthotopic cardiac xenografts were lost during the first 48 hours after transplantation, primarily caused by perioperative cardiac xenograft dysfunction (PCXD). To decrease the rate of PCXD, we adopted a preservation technique of cold non-ischemic perfusion for our ongoing pig-to-baboon cardiac xenotransplantation project.

Methods: Fourteen orthotopic cardiac xenotransplantation experiments were carried out with genetically modified juvenile pigs (GGTA1- KO/hCD46/hTBM) as donors and captive-bred baboons as recipients. Organ preservation was compared according to the two techniques applied: cold static ischemic cardioplegia (IC; n = 5) and cold non-ischemic continuous perfusion (CP; n = 9) with an oxygenated albumin-containing hyperoncotic cardioplegic solution containing nutrients, erythrocytes and hormones. Prior to surgery, we measured serum levels of preformed anti-non-Gal-antibodies. During surgery, hemodynamic parameters were monitored with transpulmonary thermodilution. Central venous blood gas analyses were taken at regular intervals to estimate oxygen extraction, as well as lactate production. After surgery, we measured troponine T and serum parameters of the recipient's kidney, liver and coagulation functions.

Jan-Michael Abicht, Paolo Brenner contributed equally to this work.

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Results: In porcine grafts preserved with IC, we found significantly depressed systolic cardiac function after transplantation which did not recover despite increasing inotropic support. Postoperative oxygen extraction and lactate production were significantly increased. Troponin T, creatinine, aspartate aminotransferase levels were pathologically high, whereas prothrombin ratios were abnormally low. In three of five IC experiments, PCXD developed within 24 hours. By contrast, all nine hearts preserved with CP retained fully preserved systolic function, none showed any signs of PCXD. Oxygen extraction was within normal ranges; serum lactate as well as parameters of organ functions were only mildly elevated. Preformed anti-non-Gal-antibodies were similar in recipients receiving grafts from either IC or CP preservation.

Conclusions: While standard ischemic cardioplegia solutions have been used with great success in human allotransplantation over many years, our data indicate that they are insufficient for preservation of porcine hearts transplanted into baboons: Ischemic storage caused severe impairment of cardiac function and decreased tissue oxygen supply, leading to multi-organ failure in more than half of the xenotransplantation experiments. In contrast, cold non-ischemic heart preservation with continuous perfusion reliably prevented early graft failure. Consistent survival in the perioperative phase is a prerequisite for preclinical long-term results after cardiac xenotransplantation.

KEYWORDS
cardiac transplantation, heart preservation, perioperative cardiac xenograft dysfunction, xenotransplantation

1 | INTRODUCTION

Cardiac allotransplantation is the best available treatment option for patients with severe terminal heart disease. Organ demand, however, exceeds the available supply.[1,2] Xenotransplantation of genetically modified porcine organs might offer a solution to the existing organ shortage.[3] Recently, we reported on non-human primates which, after having received genetically modified porcine heart replacements, survived consistently for more than 3 months,[4] a period proposed as one prerequisite before initiating a clinical trial.[5] In this context, a key challenge to be mastered is donor organ preservation to overcome primary graft failure.

For organ preservation, antegrade flushing of the coronary arteries with cardioplegic solution followed by ischemic storage at 4°C is the standard for clinical heart transplantation.[6] In this context, ischemia lasting <4 hours is associated with higher survival rates, whereas ischemic preservation of more than 6 hours is a risk factor for increased mortality within 1 year.[1] Hence, better organ protection is necessary to reduce cardiac metabolism and prevent ischemia-reperfusion injury.[7] Although a variety of clinical cardioplegic solutions have been used,[8] analyses of their effectiveness yielded controversial results,[6] suggesting that none of the currently available compounds had a clear advantage when compared to others. At present, histidine-tryptophan-ketoglutarate (HTK), University of Wisconsin (UW), and Celsior solutions are commonly used for preservation in human allotransplantation.[6,8]

With ischemic preservation, perioperative graft dysfunction occurs in 3.8%-7.4% of human allotransplantations.[9,10] After orthotopic cardiac xenotransplantation, however, early graft failure is much more frequent, posing a barrier to consistent survival since mortality has been reported to be within the range of 40%-60%.[11] This phenomenon has been coined "perioperative cardiac xenograft dysfunction" (PCXD).

Recently, Steen et al[12] reported successful orthotopic allotransplantion of pig hearts after 24 hours of preservation. They perfused donor hearts with an oxygenated 8°C cold hyperoncotic cardioplegic nutritive solution that contained hormones and erythrocytes. After explantation, the grafts were stored in a heart preservation system, where they were further perfused for 24 hours with the same solution before being transplanted into a recipient pig. After transplantation all recipient pigs were stable with normal blood pressures without inotropic support, normal urine production, and normal blood gases, indicating adequate organ perfusion.[12] In another study using the same method of organ preservation, the porcine hearts kept normal coronary artery endothelium-dependent relaxation and myocardial contractility without edema formation.[13] Very recently, a phase 2 trial demonstrated the feasibility and safety of non-ischemic heart preservation in human heart transplantation.[14]

To improve graft survival in xenotransplantation, we applied the Steen method to our studies by perfusing the donor hearts with the same specific solution for donor organ explantation, followed by immediate continuous perfusion; during implantation, the donor organs were perfused intermittently.

The primary findings of our recent experiments in orthotopic cardiac xenotransplantation were published elsewhere.[4] Here, we will focus on the immediate outcomes of this approach and discuss the effect of non-ischemic heart preservation on PCXD.

2 | METHODS

2.1 | Animals

Hearts from 14 juvenile pigs (German Landrace/Large White), homozygous for alpha-1,3-galactosyltransferase knockout (GGTA1-KO), and hemizygous transgenic for human CD46 (hCD46) and human thrombomodulin (hTBM) (Revivicor, Blacksburg, USA, and Institute of Molecular Animal Breeding and Biotechnology Gene Center, LMU Munich, Germany) were transplanted into male captive-bred baboons (*Papio anubis*; German Primate Centre DPZ). Localization and stability of hCD46 and hTBM expression were verified post-mortem.[4]

2.2 | Anesthesia

Transthoracic echocardiographic examinations of the donor pigs were carried out 1 week prior to surgery to exclude congenital heart defects. All animals were fasted for 12 hours prior to anesthesia. Following intramuscular premedication, general anesthesia of both baboons and pigs was induced with intravenous bolus administrations of propofol (Propofol-Lipuro 2%, B. Braun Melsungen AG) and fentanyl (Fentanyl-Janssen; Janssen-Cilag GmbH). Anesthesia was maintained with either continuous infusions of propofol (0.1-0.2 mg/kg/min) or inhalation of sevoflurane (1-2 vol% endexpiratory; sevorane, AbbVie Germany GmbH & Co. KG). Analgesia was maintained with repetitive bolus administrations of fentanyl (2.5-8 µg/kg every 30-45 minutes). After endotracheal intubation, the animals were mechanically ventilated. During anesthesia, ECG and blood pressure were monitored and ventilation adjusted to end-tidal CO_2 as necessary. Post-operatively, transthoracic echocardiographic examinations of the transplanted graft were carried out at regular intervals.

2.3 | Surgical procedures

After median sternotomy, the donor pigs were heparinized (500 IU/kg; heparin-natrium-25000-ratiopharm, Ratiopharm GmbH). The ascending aorta was cannulated and cross-clamped. Cardioplegia was then initiated proximally, using either standard crystalloid solution or continuous non-ischemic perfusion (see below for further information). The porcine hearts were excised and stored until needed for implantation.

Median sternotomy of the recipient baboon was performed in general anesthesia. Following heparinization (500 IU/kg), the ascending aorta and both venae cavae were cannulated and connected to the heart-lung machine. Cardiopulmonary bypass (CPB) commenced, at 34°C the ascending aorta was cross-clamped, and the donor heart transplanted using the technique of Shumway and Lower.[15]

TABLE 1 Composition of the perfusion medium used for continuous non-ischemic preservation of donor hearts, as described by Steen et al[12]

Na^+	136 mmol/L
K^+	23 mmol/L
Ca^{2+}	1.3 mmol/L
Mg^{2+}	8.0 mmol/L
Cl^-	142 mmol/L
HCO_3^-	25 mmol/mL
PO_4^{2-}	1.3 mmol/L
D-Glucose	6.3 mmol/L
Albumin	75 g/L
Cocaine	6 nmol/L
Noradrenaline	6 nmol/L
Adrenaline	6 nmol/L
T3	3 nmol/L
T4	2 nmol/L
Cortisol	420 nmol/L
Insulin	8 U/L
Imipenem	20 mg/L
Erythrocytes (Hct)	10%-15%
96% O_2 + 5% CO_2	0.2 L/min

2.4 | Donor heart preservation

Static ischemic heart preservation (IC, ischemic cardioplegia) was initiated using 20 mL/kg 4°C unmodified crystalloid cardioplegic solutions, either Custodial HTK (Dr F. Köhler Chemie GmbH) or Belzer UW solution (Preservation Solutions Inc). Following excision, the heart was submersed in cardioplegic solution and stored on ice.

Non-ischemic continuous preservation (CP) was initiated using antegrade aortic perfusion at 20 mm Hg with 600 mL 8°C cold preservation medium (oxygenated albumin-containing hyperoncotic cardioplegic solution with hormones, erythrocytes, and additional ingredients as shown in Table 1 Ref. 12). The asystolic heart was then excised and a large cannula was inserted into the ascending aorta. Continuous perfusion with the preservation medium was then provided by an extracorporeal heart preservation system consisting of a pressure- and flow-controlled roller pump, an O_2/CO_2-exchanger, a

leukocyte filter, an arterial filter, and a cooler/heater unit as described elsewhere in more detail.[12] To prevent left ventricular dilation during perfusion, the mitral valve was made incompetent using a plastic tube. The heart was then submersed into the heart-lung-machine's reservoir filled with approximately 3 L of preservation medium at a temperature of 8°C. During preservation, the organ was continuously perfused at a pressure of 20 mm Hg; during implantation, perfusion was changed to an intermittent mode for 2 minutes every 15 minutes.

2.5 | Immunosuppression

An immunosuppressive regimen based on Mohiuddin was used.[16] In short, all animals received B cell–depleting anti-CD20 antibody (MabThera; Roche Pharma AG), ATG (Thymoglobulin; Sanofi-Aventis GmbH), and either anti-CD40 monoclonal antibody (mouse/rhesus chimeric IgG4 clone 2C10R4, NIH Non-human Primate Reagent Resource, MassBiologicalsUSA; courtesy of K. Reimann) or humanized αCD40L PAS Fab (XL-protein GmbH and Wacker-Chemie) for induction therapy. Immunosuppression was maintained with mycophenolate mofetil (CellCept, Roche), either anti-CD40 monoclonal or αCD40L PASylated Fab, and methylprednisolone (Urbason soluble, Sanofi-Aventis, Germany GmbH).

2.6 | Hemodynamic monitoring of the recipient

For continuous hemodynamic measurements, a central venous catheter was placed into the internal jugular vein (Arrow International), an arterial catheter (Thermodilution Pulsiocath; Pulsion Medical Systems) into the right femoral artery. Cardiac output (CO) was assessed by transpulmonary thermodilution and indexed to body surface area using the formula $0.083 \times B^{0.639}$, where B is body weight in kg[17] to obtain the cardiac index (CI). Hemodynamic measurements were done in triplets at a steady state: after induction of anesthesia, one hour after weaning from CPB, after closing the chest, and before removing the arterial catheter. Data were recorded with PiCCOWin software (Pulsion Medical Systems).

2.7 | Blood gas analyses and laboratory tests

Central venous oxygen saturation ($s_{cv}O_2$ [%]) and lactate [mmol/L] were measured before surgery and at regular intervals after weaning from CPB (RAPIDlab® 1265, Siemens). Blood samples testing major organ functions were taken in the morning of the first post-operative day or before euthanasia, if the experiment had to be terminated earlier.

2.8 | Anti-non-Gal antibody titers of the recipient

Preoperative plasma titers of anti-non-Gal IgM and IgG antibodies were measured by flow cytometry following the consensus protocol published previously[18] and described in more detail elsewhere.[4] In brief, aortic endothelial cells of genetically modified pigs (GTTA1-KO/hCD46/hTBM) were incubated with diluted baboon plasma. After washing with cold staining buffer, the cells were incubated with goat anti-human IgM-RPE (SouthernBiotech) or goat anti-human IgG-FITC (Thermo Fisher Scientific). After rewashing and resuspending, fluorescence was acquired on FACS LSRII (BD Biosciences). Data were analyzed using FlowJo software for detection of mean fluorescence intensity in the FITC or RPE channel.

2.9 | Statistics

Data analysis was performed with GraphPad Prism 8.0 (GraphPad Software Inc). For analysis of two independent factors in repeatedly measured animals, a mixed model using a compound symmetry covariance matrix which is fit using restricted maximum likelihood was applied. This mixed model can manage missing values in contrast to a repeated measures (RM) two-way ANOVA. In the absence of missing values, this method gives the same p values and multiple comparisons tests as RM ANOVA. In the presence of missing values (missing completely at random), the results can be interpreted like a RM ANOVA. Statistically significant differences between two independent groups were determined using unpaired two-sided Student's *t* tests. Data are presented as single measurements with group means ± standard deviations (SD). $P < .05$ was considered significant.

2.10 | Study approval

The study was carried out according to the European Law on Protection of Animals for Scientific Purposes and was approved by the Government of Upper Bavaria, Germany. Care of the animals was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, 1985) and the German Law for the Care of Experimental Animals (German Legislation for the Welfare of Laboratory Animals, article 5, §7 - §9a, revised 2006).

3 | RESULTS

3.1 | Initial donor graft function

Table 2 summarizes perfusion methods, perfusion times, initial graft function, and survival times of the individual experiments. For IC, HTK solution (n = 3) and UW solution (n = 2) were used as cardioplegia. Mean total ischemia time in this group was 123 ± 7 minutes. All organs preserved with HTK showed severely impaired function after CPB and the grafts failed within 24 hours; two animals could not be weaned from respirator due to hemodynamic instability. Organs preserved

TABLE 2 Overview of preservation method, ischemic times, continuous and intermittent perfusion times, initial function, suture-extubation time, and survival of cardiac grafts after orthotopic xenotransplantation

ID	Preservation method	Ischemia time (min)	Perfusion time (min)		Initial graft function	Suture-extubation (min)	Post-operative survival (d)
			Continuous	Intermittent			
16752	IC (UW)	124	n/a	n/a	Moderately impaired	377	3
16754	IC (HTK)	128	n/a	n/a	Severely impaired	Not extubated	1
16755	IC (UW)	112	n/a	n/a	Good	264	30
16751	IC (HTK)	121	n/a	n/a	Severely impaired	785	1
16753	IC (HTK)	128	n/a	n/a	Severely impaired	Not extubated	1
16048	CP	n/a	44	114	Good	256	18
17138	CP	n/a	85	178	Good	320	4
17140	CP	n/a	94	105	Good	326	27
17139	CP	n/a	91	112	Good	296	40
17187	CP	n/a	96	99	Good	207	51
17290	CP	n/a	86	94	Good	254	90
17186	CP	n/a	124	120	Good	414	90
17493	CP	n/a	161	87	Good	390	195
17491	CP	n/a	136	94	Good	293	182

Abbreviations: CP, continuous perfusion; HTK, histidine-tryptophan-ketoglutarate; IC, ischemic cardioplegia; UW, University of Wisconsin.

TABLE 3 Catecholamine support before, during, and after orthotopic xenotransplantation of porcine hearts preserved with either ischemic cardioplegia or continuous perfusion. Data presented as mean ± SD

Parameter	Time point	Ischemic cardioplegia		Continuous perfusion	
		Mean ± SD	n	Mean ± SD	n
Noradrenalin [μg/kg/min]	Weaning from CPB	0.14 ± 0.05	5	0.07 ± 0.06	9
	1 h after CPB	0.12 ± 0.05	5	0.02 ± 0.02	9
	2 h after CPB	0.10 ± 0.06	5	0.02 ± 0.02	9
	3 h after CPB	0.08 ± 0.08	5	0.02 ± 0.03	9
Adrenalin [μg/kg/min]	Weaning from CPB	0.05 ± 0.04	5	0.04 ± 0.02	9
	1 h after CPB	0.15 ± 0.08	5	0.04 ± 0.05	9
	2 h after CPB	0.18 ± 0.14	5	0.02 ± 0.03	9
	3 h after CPB	0.30 ± 0.38	5	0.02 ± 0.03	9

with UW solution showed good (ID 16755) or moderately impaired (ID 16752) function. In the case of ID 16755, cardiac function remained unchanged during the first 72 hours, whereas experiment ID 16752 had to be terminated after 72 hours due to multi-organ failure.

In CP-preserved organs, mean out-of-body organ perfusion times were 213 ± 35 minutes, of which continuous perfusion time inside the preservation system accounted for 102 ± 34 minutes. Organ preparation and anastomosis time was similar to that of IC-preserved hearts (111 ± 27 minutes; $P = .3920$). In contrast to the IC group, all CP animals were successfully extubated and suture-extubation time was significantly shorter (median 785 minutes vs 296 minutes; $P = .0276$, log-rank test). Organ function remained excellent throughout the first 72 hours in all experiments.

Collectively, these data suggest that CP is superior to IC in this xenotransplantation setting.

3.2 | Catecholamine support in transplanted baboons

All animals needed catecholamine support for several hours after weaning from CPB (Table 3, Figure 1A). Noradrenaline, a primarily vasopressor agent administered to counteract vasodilation caused by anesthesia, extracorporeal circulation, and ischemia-reperfusion injury, was gradually reduced over time in both groups ($F(3,36) = 5.679$, $P = .0027$). However, vasopressor demand after

CPB was significantly higher in animals with IC-preserved grafts, indicating that ischemia-reperfusion injury was more pronounced when compared to CP preservation ($F(1,12) = 15.14$, $P = .0021$).

All animals were treated with adrenaline after weaning from CPB to improve cardiac contractility (Table 3, Figure 1B). Overall, IC-preserved hearts needed up to 15 times more inotropic support than CP-preserved hearts ($F(1,12) = 7.724$, $P = .0167$). We also observed a significant interaction between the preservation techniques and time ($F(3,36) = 4.184$, $P = .0122$): IC-preserved hearts required increasing doses of adrenaline, whereas inotropic support was reduced in recipients with CP-preserved hearts. This indicates early deterioration of systolic function in the IC group, in contrast to quick recovery from CPB in the CP group. Notably, hearts preserved with UW solution needed little inotropic support (Figure 1B, gray).

3.3 | Hemodynamics of transplanted baboons

As the hemodynamic parameter of global cardiac performance, CI was measured before, during, and after surgery (Table 4 and Figure 2). In both groups, CI was similar before transplantation. After orthotopic transplantation, CI decreased in both groups ($F(3,30) = 10.58$, $P < .0001$). However, CP-preserved hearts performed significantly better in the immediate post-operative period than IC-preserved hearts $F(1,12) = 12.96$, $P = .0036$). Animals with IC-preserved hearts had a pathologically low CI after transplantation despite increasing inotropic support, indicating severe systolic heart failure, and a decreasing ability to maintain adequate organ perfusion. In these recipients, the marginal CI did not recover after surgery. By contrast, those with CP-preserved hearts had CIs that returned to normal levels within several post-operative hours ($F(3,30) = 3.753$, $P = .0212$).

3.4 | Blood gas analyses

Central venous oxygen saturation measurements were taken to assess the compensatory increase of oxygen extraction from the blood when tissue oxygen delivery is insufficient. Table 3 and Figure 3 show $S_{cv}O_2$ levels before, during, and after the transplantation procedure.

In both groups, $S_{cv}O_2$ was similar before transplantation. Along with the drop of CI, $S_{cv}O_2$ significantly decreased after transplantation in all recipients ($F(4,42) = 26.21$, $P < .0001$). In animals with CP-preserved organs, this decrease was small and $S_{cv}O_2$ measurements remained within acceptable ranges. In contrast, animals that received an IC-preserved graft showed highly pathological levels of $S_{cv}O_2$, indicating an excessive oxygen extraction from arterial blood ($F(1,12) = 41.54$, $P < .0001$). In the presence of systolic heart failure, as described by reduced CI, this indicates insufficient tissue oxygen delivery.

After surgery, $S_{cv}O_2$ levels minimally declined, but remained stable within normal ranges in recipients with CP-preserved hearts. In animals with IC-preserved hearts, $S_{cv}O_2$ levels increased slowly, but remained pathologically low for the whole immediate post-operative period ($F(4,42) = 7.205$, $P = .0002$).

Serum lactate is considered a parameter of tissue hypoperfusion and hypoxia before, during, and after surgery (Table 4 and Figure 4). Both groups showed elevated levels of serum lactate after weaning from CPB ($F(4,45) = 23.73$, $P < .0001$). In the CP group, post-operative levels rose only moderately, whereas highly pathological levels were reached in the IC group ($F(1,12) = 22.74$, $P = .0005$), indicating severe microcirculatory hypoperfusion and hypoxia. Furthermore, serum lactate increases were transient in the animals with CP-preserved hearts and levels returned to normal within 12 hours after CPB. In contrast, there was no return to baseline in animals with IC-preserved hearts ($F(4,45) = 15.86$, $P =< 0.0001$). Notably, serum lactate levels remained also low when IC preservation was done with UW solution (Figure 4, gray).

3.5 | Laboratory analyses of organ functions

Serum levels of the cardiac enzyme troponin T were measured on the first day after orthotopic transplantation to assess cardiomyocyte damage (Table 5 and Figure 5A). Troponin T levels were almost eight times higher in the IC than in the CP group ($P = .0031$), indicating severe ischemia-reperfusion injury. Serum levels of creatinine and aspartate aminotransferase (AST), and the prothrombin ratio were measured as parameters to estimate post-operative kidney,

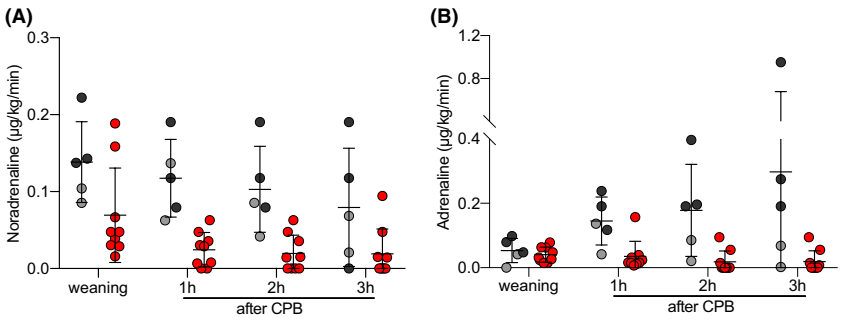

FIGURE 1 A, B, Catecholamine support (A, noradrenaline; B, adrenaline) at weaning and several hours after cardiopulmonary bypass (CPB). Donor hearts were either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data are presented as scatter plots with mean ± SD

TABLE 4 Cardiac index (CI) and central venous oxygen saturation ($S_{cv}O_2$) serum lactate levels before, during, and after orthotopic xenotransplantation of porcine hearts preserved with either ischemic cardioplegia or continuous perfusion. Data presented as mean ± SD

		Ischemic cardioplegia		Continuous perfusion	
Parameter	Time point	Mean ± SD	n	Mean ± SD	n
CI [L/min/m^2]	Before surgery	4.88 ± 0.62	5	4.97 ± 0.75	9
	1 h after CPB	3.13 ± 1.26	5	4.46 ± 0.73	8
	After surgery	2.48 ± 0.74	5	3.88 ± 0.65	8
	Catheter removal	2.70 ± 0.87	4	4.93 ± 1.61	6
$S_{cv}O_2$ [%]	Before surgery	84.6 ± 6.5	5	84.2 ± 4.9	9
	1 h after CPB	41.4 ± 26.1	3	73.3 ± 8.7	6
	6 h after CPB	40.4 ± 14.9	5	71.0 ± 11.7	9
	9 h after CPB	44.3 ± 9.1	5	70.6 ± 3.9	9
	12 h after CPB	48.5 ± 8.5	4	69.7 ± 4.2	9
Lactate [mmol/L]	Before surgery	0.82 ± 0.33	5	0.89 ± 0.22	9
	1 h after CPB	3.89 ± 3.21	5	1.45 ± 0.54	9
	6 h after CPB	8.89 ± 4.95	4	1.72 ± 0.58	9
	9 h after CPB	8.69 ± 3.18	4	2.38 ± 1.79	9
	12 h after CPB	9.373 ± 5.533	4	1.371 ± 0.898	9

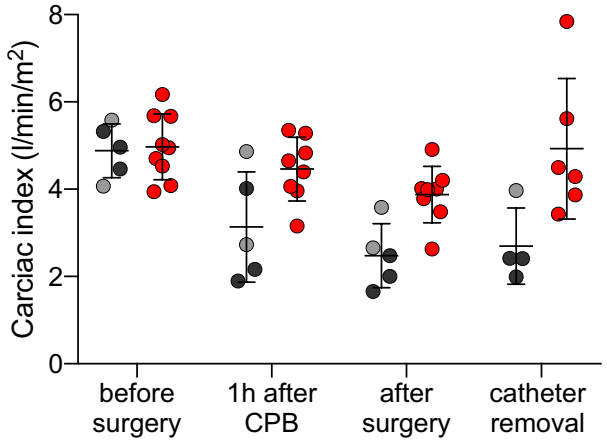

FIGURE 2 Cardiac index before and after orthotopic cardiac xenotransplantation. Donor hearts were either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data are presented as scatterplots with mean ± SD. CPB, cardiopulmonary bypass

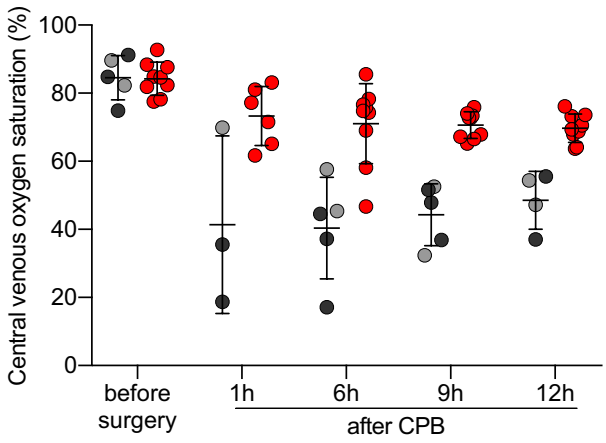

FIGURE 3 Central venous oxygen saturation ($S_{cv}O_2$) before and several hours after cardiopulmonary bypass (CPB). Donor hearts were either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data are presented as scatterplots with mean ± SD

liver, and coagulation function (Table 5). Animals with IC-preserved hearts had significantly higher serum levels of creatinine (Figure 5B; P = .0049) and AST (Figure 5C; P = .0094), and the prothrombin ratio was decreased (Figure 5D; P = .0182). When compared with the CP group, these findings indicate multi-organ dysfunction due to decreased CO of the failing graft, leading to insufficient delivery of oxygen and nutrients to the recipient's organs.

3.6 | Anti-non-Gal antibody titers

To estimate preformed baboon anti-pig antibodies (Ab) as a possible cause of early xenograft failure, plasma titers of preformed anti-non-Gal Ab were measured before transplantation (Figure 6A,B). We did not observe significant differences in IgM (P = .5765) or IgG (P = .2449) Ab titers when comparing the two groups. This indicates that levels of pre-existing antibodies against non-Gal epitopes had no influence on post-operative graft dysfunction.

4 | DISCUSSION

4.1 | Non-ischemic preservation prevents PCXD

Perioperative graft dysfunction after orthotopic cardiac xenotransplantation was successfully prevented by non-ischemic, continuous

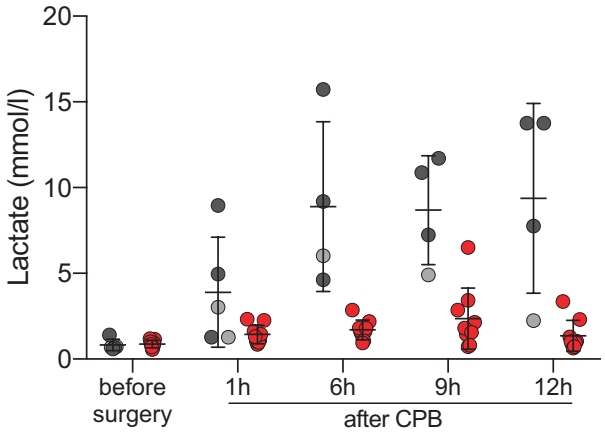

FIGURE 4 Serum lactate levels before and several hours after cardiopulmonary bypass (CPB). Donor hearts were either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data are presented as scatterplots with mean ± SD

perfusion with a hyperoncotic preservation solution containing oxygenated erythrocytes. In contrast, clinically applied crystalloid cardioplegia solutions such as HTK or UW followed by cold ischemic preservation failed to protect the xenografts from systolic pump failure, despite very short ischemia times even by clinical standards (123 ± 7 min). Under these conditions, graft failure rates of <10% would have been expected when compared to human allotransplantations.[9,10] However, three of five experiments with IC-preserved xenografts had to be terminated within 48 hours due to primary systolic failure; similar percentages have been reported by other authors.[11]

After IC preservation, 60% of the grafts failed to sustain an adequate circulation in spite of high inotropic and vasopressor support, and cardiogenic shock was the inevitable outcome shortly after CPB. Increased oxygen extraction could not compensate for insufficient tissue oxygen delivery, resulting in microcirculatory hypoxia and, eventually, multi-organ failure. Overall, continuous non-ischemic-graft preservation proved to be superior: cardiac function and tissue oxygenation were normal; all nine grafts showed good function, and there were no signs of organ dysfunction in the first 48 hours after transplantation.

Various experimental data suggest that pig hearts are especially susceptible to cold ischemia. Porcine grafts preserved with cold crystalloid solution and ischemic storage showed failure rates of 60%-100% within 48 hours after cardiac allotransplantations.[19,20] In a recent study, more than 40% of recipient pigs did not survive 24 hours when HTK was used as cardioplegic solution before proceeding with orthotopic allotransplantations.[21] Hemodynamic measurements in the surviving pigs revealed a decrease of CO by half when compared to baseline measurements, an observation we also made in the IC group. In these allotransplantation studies, the incidence and extent of early systolic graft failure was similar to that of PCXD.

In contrast, when preserving porcine hearts with non-ischemic continuous perfusion, graft function was maintained both after allo- [12] and xenotransplantation, as our data demonstrate. We believe that a combination of several factors is responsible for these superior results: (a) By adding oxygenated erythrocytes to the perfusion solution, myocardial ischemia is absent during storage and minimized during implantation. (b) The use of hypothermia and cardioplegic solution further reduces the graft's metabolic needs. (c) Continuous perfusion allows constant delivery of nutrients and removal of toxic metabolites. (d) High oncotic pressure in the preservation medium inhibits edema formation; strict pressure- and flow-controlled coronary perfusion helps to reduce capillary damage and aggravation of myocardial edema. (e) Lastly, physiological levels of catecholamines, cortisol, and thyroid hormones are substituted in the solution to maintain myocardial energy stores and cardiac function after cold preservation[22,23]; cocaine blocks the re-uptake of catecholamines into the nerve terminals, thereby ensuring physiological concentrations around alpha- and beta-receptors in the graft.[13]

TABLE 5 Laboratory parameters of organ function after orthotopic cardiac xenotransplantation

		Ischemic cardioplegia		Continuous perfusion	
Parameter	**Time point**	**Mean ± SD**	**n**	**Mean ± SD**	**n**
Troponin T [ng/mL]	Before surgery	0.013 ± 0.000	4	0.016 ± 0.006	9
	1 d after surgery	12.410 ± 8.959	4	1.597 ± 1.098	9
Creatinine [mg/dL]	Before surgery	0.86 ± 0.09	5	0.93 ± 0.19	9
	1 d after surgery	2.42 ± 0.77	5	1.20 ± 0.56	9
AST [U/l]	Before surgery	29 ± 8	5	46 ± 24	9
	1 d after surgery	1299 ± 793	5	459 ± 207	9
Prothrombin ratio [%]	Before surgery	103 ± 4	5	94 ± 8	9
	1 d after surgery	30 ± 19	5	63 ± 22	9

Donor hearts were either preserved with static ischemic cardioplegia or continuous perfusion. Data presented as mean ± SD.

AST, aspartate aminotransferase.

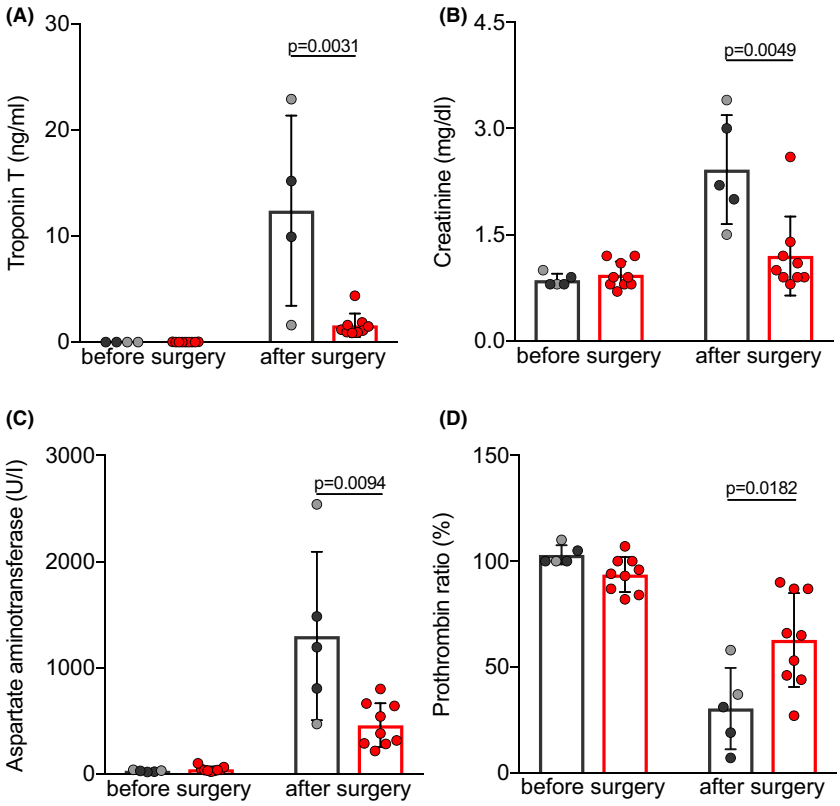

FIGURE 5 A-D, Serum levels of parameters for cardiac (A), kidney (B), liver (C), and coagulation system (D) function before surgery and on the day after orthotopic cardiac xenotransplantation. Donor hearts were either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data presented as bar plots with mean ± SD, with individuals shown as dots. Two-sided unpaired t tests, *P* values as indicated

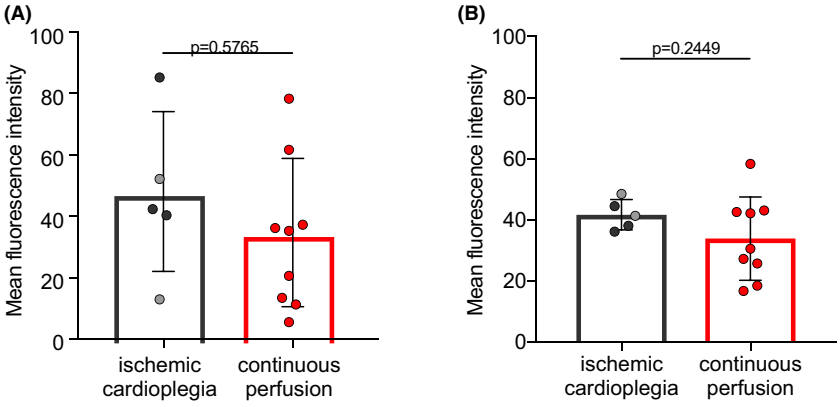

FIGURE 6 A,B Preoperative serum levels of baboon IgM (A) and IgG (B) anti-non-Gal antibodies of recipients which received porcine hearts either preserved with static ischemic cardioplegia (IC, black [histidine-tryptophan-ketoglutarate solution] and gray [University of Wisconsin solution]) or continuous perfusion (CP, red). Data presented as bar plots with mean ± SD, with individuals shown as dots. Two-sided unpaired t tests, *P* values as indicated

4.2 | Hemodynamic interspecies differences

We recently showed that the systemic vascular resistance (SVR) is twice as high in anesthetized baboons as compared to size-matched, juvenile piglets.[24] Elevated pulmonary vascular resistance (PVR) is an accepted risk factor for right ventricular failure after heart transplantation,[25,26] but arterial hypertension and increased SVR have not been associated with post-operative graft dysfunction in humans.[10] Siepe et al reported that SVR and PVR increased 2-fold 24 hours after porcine allotransplantation.[21] This group concluded that in the absence of diminished contractility, vascular resistances may be the main cause of myocardial dysfunction.

To our understanding, the function of our IC-preserved porcine grafts was severely damaged by prolonged ischemia-reperfusion injury, resulting in myocardial stunning with reduced contractility ("stone heart"). Although hearts have the potential to recover after ischemia-reperfusion injury, such dysfunctional grafts will initially have difficulties providing a sufficient CO. When confronted with a species-specific afterload mismatch, the donor hearts might not be able to cope with the increased workload, eventually leading to early systolic graft failure. Histology of the failed IC grafts is consistent with changes caused by ischemia-reperfusion damage, inadequate cardioplegia or inotropic agents (Figure 7): multiple small foci of myocyte injury with homogenization and hypereosinophilia of the

sarcoplasm, but no signs of acute cellular or hyperacute rejection nor inflammatory infiltrate.

With CP preservation, ischemia-reperfusion injury is nearly absent, allowing for optimal graft function and coping with the baboon's high-pressure cardiovascular system.

4.3 | Anti-non-Gal antibodies and thromboregulation incompatibilities

Genetically modified pigs with GGTA1-KO are not prone to hyperacute rejections caused by preformed antibodies against galactose-α-1,3-galactose. However, residual preformed anti-non-Gal antibodies bind to GGTA1-KO pig cells and are widely present in humans and non-human primates such as baboons.[27] Anti-non-Gal antibodies have been reported to contribute to early and late graft rejections and are likely the primary cause of delayed xenograft rejection.[28-31] In our study, we did not observe any differences in preoperative plasma levels of anti-non-Gal IgM or IgG antibodies between the two groups. Thus, the complete absence of any case of PCXD in the CP group suggests that anti-non-Gal antibodies did not cause PCXD in the IC group.

Ischemia-reperfusion injury is believed to be initiated by recognition of neo-epitopes exposed on injured cells, leading to activation of the complement system and acute inflammation, ultimately resulting in tissue injury.[32] This mechanism might be aggravated by incompatibilities between the porcine and primate coagulation and complement systems. In our study, donor genetic modifications were identical in both groups; all donor animals were hemizygous transgenic for hCD46 and hTBM, which have been shown to reduce any type of rejection reaction as well as thrombotic microangiopathy.[33,34] As we did not observe any case of early graft failure in the CP group, it is unlikely that complement or coagulation incompatibilities may cause PCXD in absence of ischemia-reperfusion injury.

4.4 | Differences in cardioplegic solutions for ischemic preservation

We observed differences in immediate outcome depending on the cardioplegia solution applied. However, as only a small number of experiments has been done with either solution, these observations may be arbitrary. Hearts preserved with HTK solution showed poor overall results; hearts preserved with UW solution seemed to perform better, as indicated by lower catecholamine support, postoperative oxygen extraction, and serum lactate levels. One baboon (ID 16755) was the longest survivor in this group, whereas the other one (ID 16752) worsened when conferred to his cage, requiring high doses of catecholamines over several hours. In the following 48 hours, graft function fully recovered. At this point, multi-organ dysfunction had already developed, and the experiment was terminated. In a recent meta-analysis in human allotransplantation, UW was associated with better survival as compared to HTK.[35] Steen et al[12] stressed the importance of a hyperoncotic preservation solution for organ storage to prevent myocardial edema during perfusion of cardioplegic hearts. Containing large amounts of the colloid hydroxyethyl starch, UW might be the better IC solution for ischemia-sensitive pig organs. However, no amount of colloid active substances can prevent ischemia, only oxygen can.

4.5 | Limitations

Tissue sampling was not possible immediately at the time points of hemodynamic data collection. Myocardial biopsies would have given detailed insights into histological and molecular changes but would have endangered the outcome of the experiment (bleeding, perforation).

Blood gas analyses and hemodynamic measurements were not performed in all experiments at all times due to premature

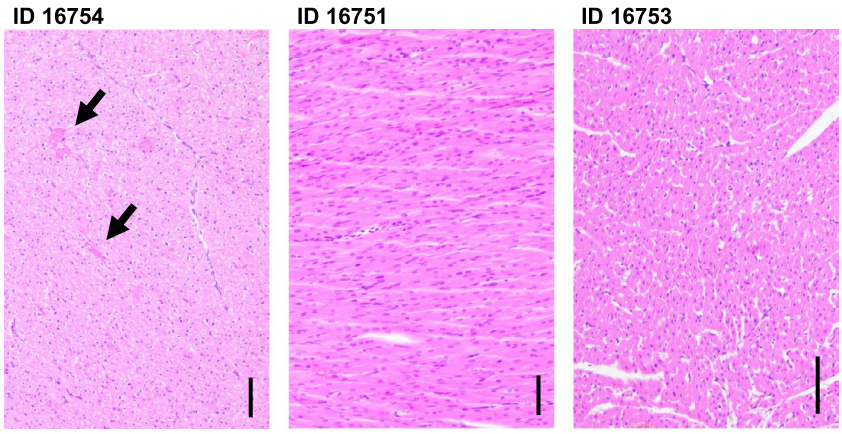

FIGURE 7 Histopathologic evaluation of the left ventricle in ID 16754, ID 16751, and ID 16753 showed largely unremarkable myocardial tissue. There were multiple small foci of myocyte injury with homogenization and hypereosinophilia of the sarcoplasm and fraying of the myocyte border as exemplarily shown for PAV56 (arrows). These changes are consistent with myocardial ischemic changes possibly caused by inotropic agents, ischemia-reperfusion damage, or inadequate cardioplegia. Noticeably, no signs of acute cellular or hyperacute rejection were present, especially no inflammatory infiltrate. Scale bars indicate 100 μm

termination or technical reasons. We tried to compensate for missing values by applying a mixed model for data analysis.

During preservation, the CP-preserved hearts received several substances which IC-preserved hearts did not, such as thyroid hormones and catecholamines (Table 1). We did not try to improve standard cardioplegic solutions (HTK, UW) for xenotransplantation purposes; with such optimized cardioplegic solutions, more consistent survival after orthotopic cardiac xenotransplantation might also be feasible. At the moment, CP preservation is about 100-fold more expensive than IC preservation; in our opinion, however, IC preservation of porcine hearts will always carry a higher risk of systolic pump failure, thus jeopardizing both the outcome of animal experiments and the future success of clinical cardiac xenotransplantation.

5 | CONCLUSION

Cold non-ischemic heart preservation with continuous perfusion guarantees optimal xenograft function in the early post-operative period after orthotopic cardiac pig-to-baboon xenotransplantation and thus prevents from PCXD. Hence, consistent long-term survival as a prerequisite for clinical application of xenotransplantation is possible.

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CONFLICT OF INTEREST

David Ayares is chief executive officer and chief scientific officer of Revivicor Inc. The other authors have no conflicts of interest to disclose.

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LETTER

Corrected: Author Correction

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Consistent success in life-supporting porcine cardiac xenotransplantation

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Heart transplantation is the only cure for patients with terminal cardiac failure, but the supply of allogeneic donor organs falls far short of the clinical need[1–3]. Xenotransplantation of genetically modified pig hearts has been discussed as a potential alternative[4]. Genetically multi-modified pig hearts that lack galactose-α1,3-galactose epitopes (α1,3-galactosyltransferase knockout) and express a human membrane cofactor protein (CD46) and human thrombomodulin have survived for up to 945 days after heterotopic abdominal transplantation in baboons[5]. This model demonstrated long-term acceptance of discordant xenografts with safe immunosuppression but did not predict their life-supporting function. Despite 25 years of extensive research, the maximum survival of a baboon after heart replacement with a porcine xenograft was only 57 days and this was achieved, to our knowledge, only once[6]. Here we show that α1,3-galactosyltransferase-knockout pig hearts that express human CD46 and thrombomodulin require non-ischaemic preservation with continuous perfusion and control of post-transplantation growth to ensure long-term orthotopic function of the xenograft in baboons, the most stringent preclinical xenotransplantation model. Consistent life-supporting function of xenografted hearts for up to 195 days is a milestone on the way to clinical cardiac xenotransplantation[7].

Xenotransplantation of genetically multi-modified α1,3-galactosyltransferase-knockout pig hearts that express human CD46 and thrombomodulin (blood group 0) was performed using the clinically approved Shumway's orthotopic technique[8]. Fourteen captive-bred baboons (*Papio anubis*, blood groups B and AB) served as recipients. All recipients received basic immunosuppression, similar to that described previously[5]: induction therapy included anti-CD20 antibody, anti-thymocyte-globulin, and the monkey-specific anti-CD40 mouse/rhesus chimeric IgG4 monoclonal antibody (clone 2C10R4)[9] or our own humanized anti-CD40L PASylated (conjugated with a long, structurally disordered Pro-Ala-Ser amino acid chain) antigen-binding fragment (Fab)[10]. During maintenance therapy methylprednisolone was reduced gradually, whereas mycophenolate mofetil and anti-CD40 monoclonal antibody or anti-CD40L PASylated Fab treatment remained constant (Extended Data Table 1). Postoperative treatment of the recipients has been described elsewhere[11].

In group I ($n = 5$), donor organs were preserved with two clinically approved crystalloid solutions (4 °C custodiol HTK (histidine-tryptophan-ketoglutarate) or Belzer's UW solution), each perfused after cross-clamping the ascending aorta before excision of the porcine donor organ. The hearts were kept in plastic bags filled with ice-cold solution and surrounded by ice cubes (static preservation).

The results of group I were disappointing. Despite short ischaemic preservation periods (123 ± 7 min), the animals survived for only 1 day ($n = 3$), 3 days ($n = 1$) and 30 days ($n = 1$) (Fig. 1a). The four short-term survivors were successfully taken off cardiopulmonary bypass (CPB) and three could be extubated, but all were lost due to severe systolic left heart failure in spite of a high dose of intravenous catecholamines (Extended Data Fig. 1). This so-called 'perioperative cardiac xenograft dysfunction' (PCXD)[12] has been observed in 40 to 60% of the orthotopic cardiac xenotransplantation experiments described in the literature[4]. The only 30-day survivor (which received a heart preserved with Belzer's UW solution) gradually developed left ventricular myocardial hypertrophy and stiffening, resulting in progressive diastolic left ventricular failure associated with increased serum levels of troponin T, an indicator of myocardial damage (Fig. 1b). Increased serum bilirubin levels (Fig. 1c) and several other clinically relevant chemical parameters (Table 1) indicated associated terminal liver disease. Upon necropsy, marked cardiac hypertrophy (Fig. 1e) with a thickened left ventricular myocardium and a decreased left ventricular cavity was evident (Fig. 1f).

To reduce the incidence of the PCXD that was observed in group I, we explored new ways to improve xenograft preservation. In group II ($n = 4$), the same immunosuppressive regime as in group I was used, but the pig hearts were preserved with an 8-°C oxygenated albumin-containing hyperoncotic cardioplegic solution that contained nutrition, hormones and erythrocytes[13]. From explantation until transplantation, the organs were continuously perfused and oxygenated using a heart-perfusion system. During implantation surgery, the hearts were intermittently perfused every 15 min until the aortic clamp was opened at the end of transplantation.

After non-ischaemic continuous organ perfusion (206 ± 43 min), all four baboons in group II could easily be taken off CPB, showed better graft function compared to animals in group I and required less catecholamine support (Extended Data Fig. 1). No organ was lost owing to PCXD. One experiment had to be terminated on the fourth postoperative day because of a technical failure; the other three animals lived for 18, 27 and 40 days (Fig. 1a). Echocardiography during the experiments revealed increasing hypertrophy of the left ventricular

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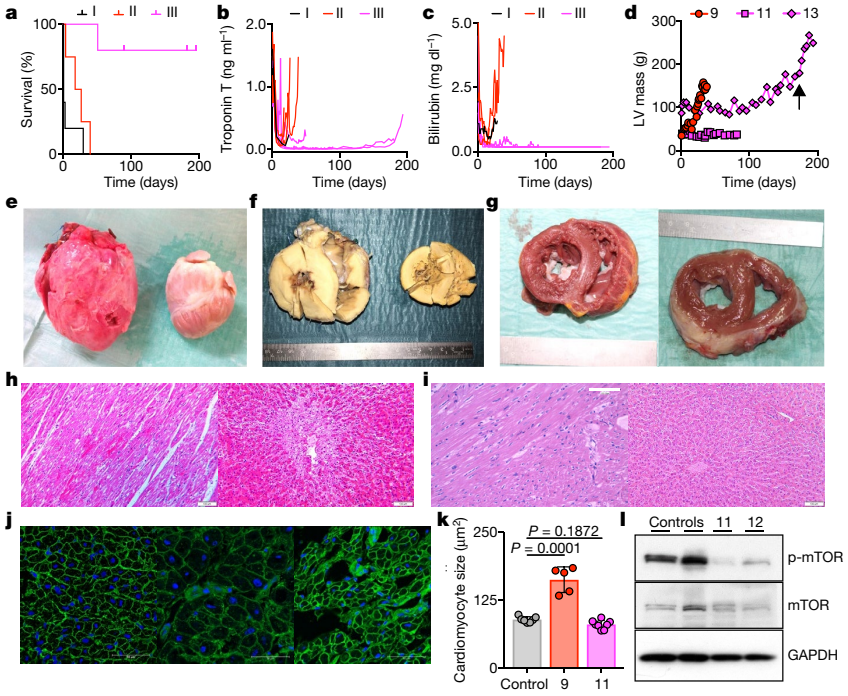

Fig. 1 | Survival, laboratory parameters, necropsy and histology after orthotopic xenotransplantation. a, Kaplan–Meier curve of survival of groups I (black; $n = 5$ animals), II (red; $n = 4$ animals) and III (magenta; $n = 5$ animals). Two-sided log-rank test, $P = 0.0007$. **b**, **c**, Serum concentrations of cardiac troponin T (**b**) and bilirubin (**c**). **d**, Left ventricular (LV) masses of xenografted hearts from animals 9 (group II), 11 and 13 (both group III); note increased graft growth after discontinuation of temsirolimus (arrow). **e**–**g**, Front view of the porcine donor heart and own heart of baboon 3 (**e**, left and right, respectively; group I) and transverse cuts of the porcine donor hearts (left) and the baboons' own hearts (right) of animals 3 (**f**) and 11 (**g**). Note the extensive left ventricular hypertrophy and reduction of left ventricular cavity of the donor organ of baboon 3 in contrast to animal 11. **h**, **i**, Haematoxylin and eosin staining of the left ventricular myocardium of the donor (left) and the liver of the recipient (right). Scale bars, 100 μm. **h**, The myocardium of animal 9 showed multifocal cell necroses with hypereosinophilia, small vessel thromboses, moderate interstitial infiltration of lymphocytes, neutrophils and macrophages. The liver of this animal had multifocal centrolobular cell vacuolizations and necroses as well as multifocal intralesional haemorrhages. **i**, The myocardium of baboon 11 had sporadic infiltrations of lymphocytes, multifocal minor interstitial oedema whereas the liver had small vacuolar degeneration of hepatocytes (lipid type). **j**, Wheat germ agglutinin-stained myocardial sections of a sham-operated porcine heart (left), and the hearts transplanted into animals 9 (centre) and 11 (right). Scale bar, 50 μm. **e**–**j**, $n = 4$, groups I/II; $n = 3$, group III; $n = 1$, control; one representative biological sample for each group is shown for group I/II, group III and control (**j**). **k**, Quantitative analysis of cardiomyocyte cross-sectional areas. Data are mean ± s.d., P values are indicated, one-way analysis of variance (ANOVA) with Holm–Sidak's multiple comparisons test ($n = 3$ biologically independent samples with 5–8 measurements each). **l**, Western blot analysis of myocardium from transplanted hearts of animals 11 and 12 showed reduced mTOR phosphorylation (p-mTOR) compared to age-matched control samples. $n = 2$, group III; $n = 2$, controls. For gel source data, see Supplementary Fig. 1.

myocardium as measured by left ventricular mass[14,15] (Fig. 1d), left ventricular stiffening and decreasing left ventricular filling volumes (Extended Data Fig. 2a). Graft function remained normal throughout the experiments, but diastolic relaxation gradually deteriorated (Supplementary Video 1). Troponin T levels were consistently above normal range and increased markedly at the end of each experiment (Table 1 and Fig. 1b) and simultaneously platelet counts decreased whereas lactate dehydrogenase (LDH) increased (Table 1 and Extended Data Fig. 3 a, b), suggesting thrombotic microangiopathy as described for heterotopic abdominal cardiac xenotransplantation[5,16]. In addition, secondary liver failure developed: increasing serum bilirubin concentrations (Fig. 1c) and a decrease in prothrombin ratio and reduction in cholinesterase indicated a reduction in liver function, while increased serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) pointed to liver damage (Table 1). At necropsy, the weight of group II hearts had more than doubled (on average 259%) compared to the time point of transplantation. Histology confirmed myocardial cell hypertrophy (Fig. 1j, k) and revealed multifocal myocardial necroses, thromboses and immune cell infiltration (Fig. 1h); in the liver, multifocal cell necroses were observed (Fig. 1h). Taken together, these alterations are consistent with diastolic pump failure and subsequent congestive liver damage resulting from massive cardiac overgrowth. However, immunofluorescence analyses of the myocardium and plasma levels of non-galactose-α1,3-galactose xenoreactive antibodies[17] did not indicate humoral rejection of the graft (Fig. 2 and Extended Data Fig. 4).

To prevent diastolic heart failure, we investigated means of reducing cardiac hypertrophy. The following modifications were made for group III ($n = 5$): recipients were weaned from cortisone at an early stage and received antihypertensive treatment (pigs have a lower systolic blood pressure than baboons, around 80 compared to approximately 120 mm Hg, respectively) and additional temsirolimus medication was used to counteract cardiac overgrowth. After heart perfusion times of 219 ± 30 min, all five animals were easily taken off CPB, comparable to group II (Extended Data Fig. 1). None of the recipients in group III showed PCXD; all reached a steady state with good heart function after four weeks. One recipient (10) developed recalcitrant pleural effusions that were caused by occlusion of the thoracic lymph duct and was therefore euthanized after 51 days. Two recipients (11, 12) lived in good health for three months until euthanasia, according to the study protocol (Fig. 1a). In these three recipients, echocardiography revealed no increase in left ventricular mass (Fig. 1d); graft function remained normal with no signs of diastolic dysfunction (Extended Data Fig. 2b and Supplementary Video 2). Biochemical parameters of heart and liver functions as well as LDH levels and platelet counts were normal or only slightly altered throughout the experiments (Table 1, Fig. 1b, c and Extended Data Fig. 3a, b), consistent with normal histology (Fig. 1i). Histology of left ventricular myocardium showed no

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Table 1 | Serum levels of liver and heart enzymes, platelet counts and prothrombin ratio at the end of experiments that lasted longer than two weeks

	Group I	Group II			Group III					
Experiment	**3**	**6**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Reference**
Bilirubin (mg dl^{-1})	1.2	0.9	2.7	4.5	0.3	0.2	0.2	0.2	0.2	≤1.2
AST (U l^{-1})	646	896	792	354	101	27	23	63	28	≤49
PR (%)	30	6	6	6	101	96	117	26	99	70–130
CHE (kU l^{-1})	1.6	1.6	1.4	1.1	2.1	9.4	14.4	7.3	7.2	4.6–11.5
Troponin T (ng ml^{-1})	0.233	0.660	1.460	1.470	0.218	0.037	0.018	0.556	0.140	≤0.014
CK total (U l^{-1})	654	636	1017	953	3053	143	66	461	96	≤189
LDH (U l^{-1})	3252	6853	2842	1627	436	311	511	962	497	≤249
Platelets (billion particles per litre)	99	101	65	29	216	202	128	271	303	150–300
Survival (days)	30	18	27	40	51	90	90	195	182	
Causes of death	Heart and liver failure	Heart and liver failure	Heart and liver failure	Heart and liver failure	SVC thrombosis, thoracic duct occlusion	Euthanasia	Euthanasia	Euthanasia	Euthanasia	

Normal reference values are given in the right-most column. Animals from groups I and II exhibited pathological biochemical alterations that correspond to heart and liver failure; platelet counts were low and LDH was elevated. By contrast, most parameters remained close to, or within, normal ranges in animals of group III. The baboon in experiment 10 had to be euthanized because of severe pleural effusions due to superior vena cava (SVC) thrombosis and occlusion of the thoracic lymph duct. The animals in experiments 11 and 12 were euthanized after reaching the study end point of 90 days, although they did not show any signs of cardiac or liver dysfunction. Animals in experiments 13 and 14 were euthanized after six months; recipient 13 showed the signs of beginning heart and liver dysfunction. CHE, cholinesterase; CK, creatine kinase; PR, prothrombin ratio.

signs of hypertrophy (Fig. 1j, k), and western blot analysis of the myocardium revealed phosphorylation levels of mTOR that were lower than non-transplanted age-matched control hearts (Fig. 1l). Similar to group II, there were no signs of humoral graft rejection in group III (Fig. 2 and Extended Data Fig. 4).

The study protocol for group III was extended aiming at a graft survival of six months. The last two recipients in this group (13, 14) were allowed to survive in good general condition for 195 and 182 days, with no major changes to platelet counts or serum LDH and bilirubin levels (Fig. 1a, c and Extended Data Fig. 3a, b). Intravenous temsirolimus treatment was discontinued on day 175 and on day 161. Up to this point, systolic and diastolic heart function was normal (Supplementary Video 3). Thereafter, increased growth of the cardiac graft was observed in both recipients (Fig. 1d), emphasizing the importance of mTOR inhibition in the orthotopic xenogeneic heart xenotransplantation model.

Similar to the changes observed in group II, the smaller recipient 13 developed signs of diastolic dysfunction, which was associated with elevated serum levels of troponin T and the start of congestive liver damage (increased serum ALT and AST levels, decreased prothrombin ratio and cholinesterase); platelet counts remained within normal ranges (Table 1, Fig. 1b, c and Extended Data Fig. 3a, b). Histology confirmed hepatic congestion and revealed multifocal myocardial necroses without immune cell infiltrations or signs of thrombotic microangiopathy. In the larger recipient 14, who had to be euthanized simultaneously with animal 13, the consequences of cardiac overgrowth were minimal.

Here we show consistent survival of life-supporting pig hearts in non-human primates for at least three months that meets the preclinical efficacy requirements for the initiation of clinical xenotransplantation trials as suggested by an advisory report of the International Society for Heart and Lung Transplantation[7].

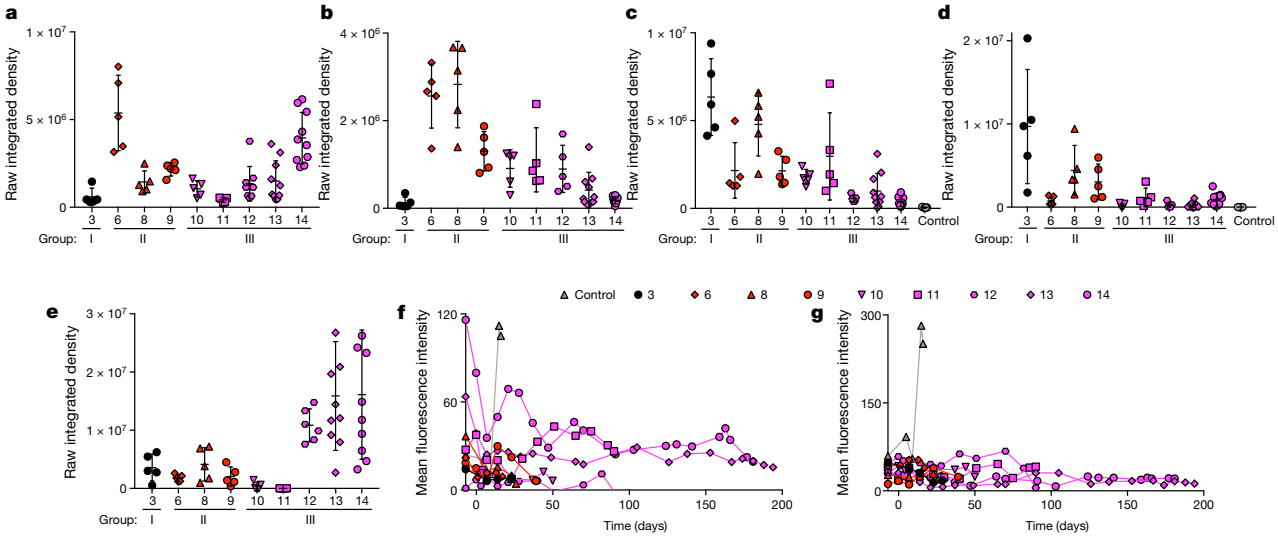

Fig. 2 | Quantitative evaluation of antibodies, complement and fibrin in myocardial tissue and serum levels of non-galactose-α1,3-galactose xenoreactive antibodies. a–e, Quantitative evaluation of fluorescence intensities ($n = 9$ biologically independent samples with 5–10 measurements per experiment; for representative images see Extended Data Fig. 4). Raw integrated densities are shown for IgM (**a**), IgG (**b**), C3b/c (**c**), C4b/c (**d**) and fibrin (**e**). Group I (animal 3), black; group II (animals 6, 8, 9), red; group III (animals 11–14), magenta. C3b/c and C4b/c values are compared to those of controls measured in healthy pig hearts. Data are mean ± s.d. **f**, **g**, Levels of non-galactose-α1,3-galactose xenoreactive IgM and IgG antibodies in baboon plasma; antibody binding to α-galactosyltransferase-knockout porcine aortic endothelial cells that express human CD46 and thrombomodulin was analysed by fluorescence-activated cell sorting. Values are expressed as mean fluorescence intensity. Animals 6, 9 and 10 received an anti-CD40L PASylated Fab, the others were treated with an anti-CD40 monoclonal antibody. Plasma from a baboon who rejected a heterotopically intrathoracic transplanted pig heart served as positive control (grey).

Two steps were key to success. First, non-ischaemic porcine heart preservation was found to be important for the survival of the xenografted hearts. Xenografted hearts from group I that underwent ischaemic static myocardial preservation with crystalloid solutions (as used for clinical allogeneic procedures) showed PCXD in four out of five cases, necessitating higher amounts of catecholamines. This phenomenon is clearly similar to 'cardiac stunning', the occurrence of which has been known since the early days of cardiac surgery and does not represent hyperacute rejection[4]. By contrast, in groups II and III (non-ischaemic porcine heart preservation by perfusion)[13], all nine recipients came off CPB easily since their cardiac outputs remained unchanged compared to baseline. The short-term results achieved in these groups were excellent even by clinical standards.

The second key step was the prevention of detrimental xenograft overgrowth. Previous pig-to-baboon kidney and lung transplantation experiments have suggested that growth of the graft depends more on intrinsic factors than on stimuli from the recipient such as growth hormones[18]. The massive cardiac hypertrophy in our group-II recipients indicates a more complex situation. Notably, a transplanted heart in this group had a 62% greater weight gain than the non-transplanted heart of a sibling in the same time span (Extended Data Fig. 2c).

In group III, cardiac overgrowth was successfully counteracted by a combination of treatments: (i) decreasing the blood pressure of the baboons to match the lower porcine levels; (ii) tapering cortisone at an early stage—cortisone can cause hypertrophic cardiomyopathy in early life in humans[19]; and (iii) using the sirolimus prodrug temsirolimus to mitigate myocardial hypertrophy. Sirolimus compounds are known to control the complex network of cell growth by inhibiting both mTOR kinases[20]. There is clinical evidence that sirolimus treatment can attenuate myocardial hypertrophy and improve diastolic pump function[21,22], as well as ameliorate rare genetic overgrowth syndromes in humans[23]. In addition to the effects of human thrombomodulin expression in the graft[5,24], temsirolimus treatment may prevent the formation of thrombotic microangiopathic lesions even further by reducing collagen-induced platelet aggregation and by destabilizing platelet aggregates formed under shear stress conditions[25].

In summary, our study demonstrates that consistent long-term life-supporting orthotopic xenogeneic heart transplantation in the most relevant preclinical model is feasible, facilitating clinical translation of xenogeneic heart transplantation.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0765-z.

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Competing interests D.A. is chief executive officer and chief scientific officer of Revivicor. Inc. A.S. and U.B. are cofounders of XL-protein GmbH, Germany. The other authors declare no competing interests.

Additional information
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METHODS

Animals. Experiments were carried out between February 2015 and August 2018. Fourteen juvenile pigs of cross-bred genetic background (German Landrace and Large White, blood group 0) served as donors for heart xenotransplantation. All organs were homozygous for α1,3-galactosyltransferase knockout (GTKO), and heterozygous transgenic for human CD46 (hCD46) and human thrombomodulin (hTM)[24] (Revivicor and Institute of Molecular Animal Breeding and Biotechnology). Localization and stability of hCD46 and hTM expression were verified post mortem by immunohistochemistry (Extended Data Fig. 5). Donor heart function and absence of valvular defects were evaluated seven days before transplantation by echocardiography. Fourteen male captive-bred baboons (*P. anubis*, blood groups B and AB) were used as recipients (German Primate Centre).

The study was approved by the local authorities and the Government of Upper Bavaria. All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health and German Legislation).

Anaesthesia and analgesia. Baboons were premedicated by intramuscular injection of ketamine hydrochloride 6–8 mg kg^{-1} (ketavet 100 mg ml^{-1}; Pfizer) and 0.3–0.5 mg kg^{-1} midazolam (midazolam-ratiopharm; Ratiopharm). General anaesthesia was induced with an intravenous bolus of 2.0–2.5 mg kg^{-1} propofol (propofol-lipuro 2%; B. Braun Melsungen) and 0.05 mg fentanyl (fentanyl-janssen 0.5 mg; Janssen-Cilag), and maintained with propofol (0.16 ± 0.06 mg kg^{-1} min^{-1}) or sevoflurane (1–2 vol% endexpiratory; sevorane, AbbVie) and bolus administrations of fentanyl (6–8 μg kg^{-1}, repeated every 45 min) as described elsewhere[11]. Continuous infusion of fentanyl, ketamine hydrochloride and metamizole (novaminsulfon-ratiopharm 1 g per 2 ml; Ratiopharm) was applied postoperatively to ensure analgesia.

Explantation and preservation of donor hearts. Pigs were premedicated by intramuscular injection of ketamine hydrochloride 10–20 mg kg^{-1}, azaperone 10 mg kg^{-1} (stresnil 40 mg ml^{-1}; Lilly Deutschland) and atropine sulfate (atropinsulfat B. Braun 0.5 mg; B. Braun Melsungen). General anaesthesia was induced with an intravenous bolus of 20 mg propofol and 0.05 mg fentanyl and maintained with propofol (0.12 mg kg^{-1} min^{-1}) and bolus administrations of fentanyl (2.5 μg kg^{-1}, repeated every 30 min).

After median sternotomy and heparinization (500 IU kg^{-1}), a small cannula was inserted into the ascending aorta, which was then cross-clamped distal of the cannula. In group I, the heart was perfused with a single dose of 20 ml kg^{-1} crystalloid cardioplegic solution at 4 °C: custodiol HTK solution (Dr. Franz Köhler Chemie) was used for the hearts for animals 2, 4 and 5, Belzer's UW solution (Preservation Solutions) was used for the hearts for animals 1 and 3. The appendices of the right and left atrium were opened for decompression. The heart was then excised, submersed in cardioplegic solution and stored on ice.

In groups II and III, hearts were preserved as described previously[13], using 3.5 l of an oxygenated albumin-containing hyperoncotic cardioplegic nutrition solution with hormones and erythrocytes at a temperature of 8 °C in a portable extracorporeal heart preservation system consisting of a pressure- and flow-controlled roller pump, an O_2/CO_2 exchanger, a leukocyte filter, an arterial filter and a cooler/heater unit.

After aortic cross-clamping, the heart was perfused with 600 ml preservation medium, excised and moved into the cardiac preservation system. A large cannula was introduced into the ascending aorta and the mitral valve was made temporarily incompetent to prevent left ventricular dilation; the superior vena cava was ligated; however, the inferior vena cava, pulmonary artery and pulmonary veins were left open for free outlet of perfusate. The heart was submersed in a reservoir filled with cold perfusion medium and antegrade coronary perfusion commenced through the already placed aortic cannula. The perfusion pressure was regulated at exactly 20 mm Hg. During implantation, the heart was intermittently perfused for 2 min every 15 min.

Implantation technique. The recipient's thorax was opened at the midline. Unfractionated heparin (500 IU kg^{-1}; heparin-natrium-25000-ratiopharm, Ratiopharm) was given and the heart–lung machine connected, using both venae cavae and the ascending aorta. CBP commenced and the recipient was cooled to 30 °C in group I, and 34 °C in groups II and III. After cross-clamping the ascending aorta, the recipient's heart was excised at the atrial levels, both large vessels were cut. The porcine donor heart was transplanted using Shumway's and Lower's technique[8].

A wireless telemetric transmitter (Data Sciences International) was implanted in a subcutaneous pouch in the right medioclavicular line between the fifth and sixth rib. Pressure probes were inserted into the ascending aorta and the apex of the left ventricle, an electrocardiogram lead was placed in the right ventricular wall.

Immunosuppressive regimen, anti-inflammatory and additive therapy. Immunosuppression was based on the previously published regimen[5], with C1 esterase inhibitor instead of cobra venom factor for complement inhibition (Extended Data Table 1). Induction consisted of anti-CD20 antibody (mabthera; Roche Pharma), ATG (thymoglobuline, Sanofi-Aventis), and either an anti-CD40 monoclonal antibody (mouse/rhesus chimeric IgG4 clone 2C10R4, NIH Non-human Primate Reagent Resource, Mass Biologicals; courtesy of K. Reimann; animals 1–3, 5, 7, 8, 11–14) or humanized anti-CD40L PASylated Fab (XL-Protein and Wacker-Chemie; animals 4, 6, 9, 10). Maintenance immunosuppression consisted of mycophenolate mofetil (CellCept, Roche; trough level 2–3 μg ml^{-1}), either the anti-CD40 monoclonal antibody (animals 1–3, 5, 7, 8, 11–14) or anti-CD40L PASylated Fab (animals 4, 6, 9, 10), and methylprednisolone (urbasone soluble, Sanofi-Aventis). Anti-inflammatory therapy included an IL-6-receptor antagonist (RoActemra, Roche), TNF inhibitor (enbrel, Pfizer) and an IL-1-receptor antagonist (Kineret, Swedish Orphan Biovitrum). Additive therapy consisted of acetylsalicylic acid (aspirin, Bayer Vital), unfractionated heparin (heparin-natrium-25000-ratiopharm, Ratiopharm), C1 esterase inhibitor (berinert, CSL Behring), ganciclovir (cymevene, Roche), cefuroxime (cefuroxim, Hikma) and epoetin beta (neorecormon 5000IU, Roche P).

Starting from 10 mg kg^{-1} per day, methylprednisolone was slowly reduced by 1 mg kg^{-1} every 10 days in group I and II; in group III, methylprednisolone was tapered down to 0.1 mg kg^{-1} within 19 days. Also in group III, temsirolimus (torisel, Pfizer) was added to the maintenance immunosuppression, administered as daily short intravenous infusions aiming at rapamycin trough levels of 5–10 ng ml^{-1}. Group III also received continuous intravenous antihypertensive medication with enalapril (Enahexal, Hexal AG, Holzkirchen, Germany) and metoprolol tartrate (Beloc, AstraZeneca), aiming at mean arterial pressures of 80 mm Hg and a heart rate of 100 b.p.m.

Haemodynamic measurements. After induction of general anaesthesia, a central venous catheter (Arrow International) was inserted in the left jugular vein and an arterial catheter (Thermodilution Pulsiocath; Pulsion Medical Systems) in the right femoral artery. Cardiac output and stroke volume were assessed by transpulmonary thermodilution and indexed to the body surface area of the recipient using the formula $0.083 \times B^{0.639}$ where B is body weight in kg. Measurements were taken after induction of anaesthesia and 60 min after termination of CPB in steady state and recorded with PiCCOWin software (Pulsion Medical Systems). All data were processed with Excel (Microsoft) and analysed with GraphPad Prism 7.0 (GraphPad Software).

Quantification of left ventricular mass, left ventricular mass increase and fractional shortening. Transthoracic echocardiographic examinations were carried out under analgosedation at regular intervals using an HP Sonos 7500 (HP) and a Siemens Acuson X300 (Siemens); midpapillary short axis views were recorded. Left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD), interventricular septum thickness at end diastole (IVSd) and posterior wall thickness at end diastole (PWd) were measured; the mean of three measurements was used for further calculations and visualization (Excel and PowerPoint, Microsoft).

Left ventricular mass was calculated using equation (1), relative left ventricular mass increase and left ventricular (LV) fractional shortening (FS) was calculated using equations (2) and (3) according to previously published methods[14,15].

$$\text{LV mass (g)} = 0.8(1.04((\text{LVEDD} + \text{IVSd} + \text{PWd})^3 - \text{LVEDD})) + 0.6 \quad (1)$$

$$\text{LV mass increase (\%)} = ((\text{LV mass}_{\text{end}}/\text{LV mass}_{\text{start}}) - 1) \times 100 \quad (2)$$

$$\text{FS (\%)} = ((\text{LVEDD} - \text{LVESD})/\text{LVEDD}) \times 100 \quad (3)$$

Necropsy and histology. Necropsies and histology were performed at the Institute of Veterinary Pathology and the Institute of Pathology. Specimens were fixed in formalin, embedded in paraffin and plastic, sectioned and stained with haematoxylin and eosin.

Histochemical analysis. Cryosections (8 μm) were generated using standard histological techniques. Cardiomyocyte size was quantified as the cross-sectional area. In brief, 8-μm thick cardiac sections of the left ventricle were stained with Alexa Fluor 647-conjugated wheat germ agglutinin (Life Technologies) and the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies). Images were acquired with a 63× objective using a Leica TCS SP8 confocal microscope; SMASH software (MATLAB, https://de.mathworks.com/products/matlab.html) was used to determine the average cross-sectional area of cardiomyocytes in one section (200–300 cells per section and 5– 8 sections per heart).

Immunofluorescence staining. Myocardial tissue biopsies were embedded in Tissue-Tek (Sakura Finetek) and stored frozen at −80 °C. For immunofluorescence staining, 5-μm cryosections were cut, air-dried for 30 to 60 min and stored at −20 °C until further analysis. The cryosections were fixed with ice-cold acetone, hydrated and stained using either one-step direct or two-step indirect immunofluorescence techniques. The following antibodies were used: rabbit anti-human C3b/c (DAKO), rabbit anti-human C4b/c-FITC (DAKO), goat anti-pig IgM (AbD

Serotec), goat anti-human IgG–FITC (Sigma-Aldrich) and rabbit anti-human fibrinogen–FITC (DAKO). Secondary antibodies were donkey anti-goat IgG–Alexa Fluor 488 (Thermo Fisher Scientific), sheep anti-rabbit Cy3 (Sigma-Aldrich). Nuclear staining was performed using DAPI (Boehringer, Roche Diagnostics). The slides were analysed using a fluorescence microscope (DM14000B; Leica). Five to ten immunofluorescence pictures per marker were acquired randomly and the fluorescence intensity was quantified using ImageJ software, version 1.50i (https://imagej.nih.gov/ij/) on unmanipulated TIFF images. All pictures were taken under the same conditions to allow correct quantification and comparison of fluorescence intensities.

Assessment of non-galactose-α1,3-galactose antibody levels. Plasma levels of non-galactose-α1,3-galactose baboon IgM and IgG antibodies were measured by flow cytometry following the consensus protocol published previously[17]. In brief, GTKO/hCD46/hTM porcine aortic endothelial cells were collected and suspended at 2×10^6 cells per ml in staining buffer (PBS containing 1% BSA). Plasma samples were heat-inactivated at 56 °C for 30 min and diluted 1:20 in staining buffer. Porcine aortic endothelial cells were incubated with diluted baboon plasma for 45 min at 4 °C. Cells were then washed with cold staining buffer and incubated with goat anti-human IgM–RPE (Southern Biotech) or goat anti-human IgG–FITC (Thermo Fisher) for 30 min at 4 °C. After rewashing with cold staining buffer, cells were resuspended in PBS, fluorescence was acquired on FACS LSRII (BD Biosciences) and data were analysed using FlowJo analysis software for detection of mean fluorescence intensity (MFI) in the FITC channel or in the RPE channel. Data were then plotted using Prism 7 (GraphPad Software).

Western blot analysis. For protein extraction, heart samples were homogenized in Laemmli sample buffer and the protein content was estimated using the bicinchoninic acid (BCA, Merck) protein assay. Then, 20 μg total protein was separated by 10% SDS–PAGE and transferred to PDVF membranes (Millipore) by electroblotting. Membranes were washed in Tris-buffered saline solution with 0.1% Tween-20 (Merck) (TBS-T) and blocked in 5% w/v fat-free milk powder (Roth) for 1 h at room temperature. Membranes were then washed again in TBS-T and incubated in 5% w/v BSA (Roth) of the appropriate primary antibody overnight at 4 °C. The following antibodies were used: rabbit anti-human p-mTOR (5536; Cell Signaling), rabbit anti-human mTOR (2983; Cell Signaling) and rabbit anti-human GAPDH (2118; Cell Signaling). After washing, membranes were incubated in 5% w/v fat-free milk powder with a horseradish peroxidase-labelled secondary antibody (goat anti-rabbit IgG; 7074; Cell Signaling) for 1 h at room temperature. Bound antibodies were detected using an enhanced chemiluminescence detection reagent (ECL Advance Western Blotting Detection Kit, GE Healthcare) and appropriate X-ray films (GE Healthcare). After detection, membranes were stripped (2% SDS, 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol) for 30 min at 70 °C and incubated with the appropriate second antibody.

Immunohistochemical staining. Myocardial tissue was fixed with 4% formalin overnight, paraffin-embedded and 3-μm sections were cut and dried. Heat-induced antigen retrieval was performed in Target Retrieval solution (S1699, DAKO) in a boiling water bath for 20 min for hCD46 and in citrate buffer, pH 6.0, in a steamer for 45 min for hTM, respectively. Immunohistochemistry was performed using the following primary antibodies: mouse anti-human CD46 monoclonal antibody (HM2103, Hycult Biotech) and mouse anti-human thrombomodulin monoclonal antibody (sc-13164, Santa Cruz). The secondary antibody was a biotinylated AffiniPure goat anti-mouse IgG (115-065-146, Jackson ImmunoResearch). Immunoreactivity was visualized using 3,3-diaminobenzidine tetrahydrochloride dihydrate (DAB) (brown colour). Nuclear counterstaining was done with haemalum (blue colour).

Statistical analysis. For survival data, Kaplan–Meier curves were plotted and the Mantel–Cox log-rank test was used to determine significant differences between groups. For haemodynamic data, statistical significance was determined using unpaired and paired two-sided Student's *t*-tests as indicated; data presented as single measurements with bars as group means ± s.d. For histochemical analysis, a one-way ANOVA with Holm–Sidak's multiple comparisons was used to determine statistical significance; data are mean ± s.d.; $P < 0.05$ was considered significant. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

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

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ORIGINAL ARTICLE

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Hemodynamic evaluation of anesthetized baboons and piglets by transpulmonary thermodilution: Normal values and interspecies differences with respect to xenotransplantation

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Abstract

Background: Transpulmonary thermodilution is well established as a tool for in-depth hemodynamic monitoring of critically ill patients during surgical procedures and intensive care. It permits easy assessment of graft function following cardiac transplantation and guides post-operative volume and catecholamine therapy. Since no pulmonary catheter is needed, transpulmonary thermodilution could be useful in experimental cardiac pig-to-baboon xenotransplantation. However, normal values for healthy animals have not yet been reported. Here, we present data from piglets and baboons before xenotransplantation experiments and highlight differences between the two species and human reference values.

Methods: Transpulmonary thermodilution from baboons (body weight 10-34 kg) and piglets (body weight 10-38kg) were analyzed. Measurements were taken in steady state after induction of general anesthesia before surgical procedures commenced. Cardiac index (CI), mean arterial pressure (MAP), systemic vascular resistance index (SVRI), parameters quantifying cardiac filling (global end-diastolic volume index, GEDI), and pulmonary edema (extravascular lung water, ELWI) were assessed.

Results: Preload, afterload, and contractility parameters clearly correlated with total body weight or body surface area. Baboons had lower CI values than weight-matched piglets (4.2 ± 0.9l/min/m^2 vs 5.3 ± 1.0/min/m^2, $P < .01$). MAP and SVRI were higher in baboons than piglets (MAP: 99 ± 22 mm Hg vs 62 ± 11 mm Hg, $P < .01$; SVRI: 1823 ± 581 dyn*s/cm^5*m^2 vs 827 ± 204 dyn*s/cm^5*m^2, $P < .01$). GEDI and ELWI did differ significantly between both species, but measurements were within similar ranges (GEDI: 523 ± 103 mL/m^2 vs 433 ± 78 mL/m^2, $P < .01$; ELWI: 10 ± 3 mL/kg vs 11 ± 2 mL/kg, $P < .01$). Regarding adult human reference values, CI was similar to both baboons and piglets, but all other parameters were different.

Andreas Bauer and Jan-Michael Abicht equally contributed to this work.

Conclusions: Parameters of preload, afterload, and contractility differ between baboons and piglets. In particular, baboons have a much higher afterload than piglets, which might be instrumental in causing perioperative xenograft dysfunction and post-operative myocardial hypertrophy after orthotopic pig-to-baboon cardiac xenotransplantation. Most transpulmonary thermodilution-derived parameters obtained from healthy piglets and baboons lie outside the reference ranges for humans, so human normal values should not be used to guide treatment in those animals. Our data provide reference values as a basis for developing algorithms for perioperative hemodynamic management in pig-to-baboon cardiac xenotransplantation.

KEYWORDS

baboon, cardiac transplantation, hemodynamic monitoring, perioperative management, reference range, transpulmonary thermodilution, xenotransplantation

1 | INTRODUCTION

Minimally invasive hemodynamic monitoring has become the standard procedure for critically ill patients undergoing surgery or requiring intensive care. Pulmonary artery catheterization for thermodilution measurement of cardiac output (CO) is still considered the gold standard, but its use has been declining in recent years.[1] Less invasive methods of transpulmonary thermodilution (TPTD), such as the PiCCO system (Pulsion Medical Systems SE), have become an important alternative, especially for children where the use of Swan Ganz catheters is limited by vessel size.[2] CO measured by TPDT has repeatedly been shown to correlate well with CO measured by pulmonary artery catheter in animal experiments, as well as clinical settings in cardiac surgery, acute lung failure, intensive care, transplantation surgery, and pediatrics.[3]

For TPTD, a central venous access and a catheter placed in the femoral artery are required. Arterial blood pressure is monitored continuously via a pressure transducer. After initiating a hemodynamic measurement with a central venous injection of cold saline solution, the thermistor at the tip of the arterial catheter measures the blood temperature change over time. Cardiac output (CO) is measured based on a modified Stewart-Hamilton algorithm.[4,5] Stroke volume (SV) and systemic vascular resistance (SVR) are calculated once heart rate (HR) and the systemic arterial pressure are obtained. Additional hemodynamic parameters are derived from the mean transit time and the exponential downslope time of the thermodilution curve. Global end-diastolic volume (GEDV) describes cardiac preload, and extravascular lung water (EVLW) quantifies pulmonary edema. To enable better comparison between individuals of different size, indexing to body surface area (BSA) is performed for parameters of cardiac output (CI, cardiac index; SVI stroke volume index), volumetric preload (GEDI), and afterload (SVRI); EVLW is indexed to total body weight (TBW). A combined interpretation of these indexed parameters allows a detailed assessment of preload, afterload, contractility, and volume status of critically ill patients, which are pre-requisites for goal-directed volume and catecholamine therapy. The reader is referred to two excellent reviews for further details of clinical and technical aspects of this topic.[6,7]

In xenotransplantation, TPTD has been used to assess CO during pig-to-primate kidney[8] and heart transplantation.[9-11] As for human allotransplantation, comprehensive hemodynamic monitoring and careful therapeutic management are key to a favorable outcome after cardiac xenotransplantation. However, little is known about the normal values in baboons and piglets of the sizes used for these experiments. To our knowledge, no reference tables are as yet available.

2 | METHODS

2.1 | Animals

Hemodynamic data sets from pigs and baboons obtained between August 2006 and October 2018 were analyzed. All baboons were captive-bred (*Papio anubis* and *Papio hamadryas*, n = 47, age 3-11 years, body weight 10-34kg; German Primate Center DPZ, Göttingen, Germany). The piglets were juveniles of the breeds large white/landrace and were "wild type" with no genetic modifications (*Sus scrofa*, n = 45, age approximately 1-4 months, body weight 10-38kg; Institute for Molecular Animal Breeding and Biotechnology, Gene Center, Faculty of Veterinary Medicine, Ludwig-Maximilians-University). The perfusion and xenotransplantation studies of which these animals were a part were carried out according to the European Law on Protection of Animals for Scientific Purposes and were approved by the Government of Upper Bavaria, Germany. Care of the animals was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, 1985) and the German Law for the Care of Experimental Animals (German Legislation for the Welfare of Laboratory Animals, article 5, §7-§9a, revised 2006).

2.2 | Anesthesia

All animals were fasted for 12 hours prior to anesthesia. General anesthesia of both baboons and pigs was induced with intravenous bolus administrations of propofol and fentanyl. Anesthesia was maintained with either continuous infusions of propofol (0.1-0.2 mg/kg/min; baboons and pigs) or inhalation of isoflurane (0.8-1.2 vol%) and sevoflurane (1-2 vol% end-expiratory concentration) for baboons only. Analgesia was maintained with repetitive bolus administrations of fentanyl (2.5-8 µg/kg every 30-45 minutes). After endotracheal intubation, the animals were ventilated mechanically. During anesthesia, ventilation was adjusted to end-tidal CO_2 as necessary, and ECG, blood pressure, and peripheral oxygen saturation were monitored.

2.3 | Invasive hemodynamic monitoring and measurements

For hemodynamic measurements, a central venous catheter was placed in the jugular vein (4Fr, 5.5Fr or 7Fr single/multi-lumen central venous catheter; Arrow International, Reading, PA, USA) and a 3 or 4Fr arterial catheter (Thermodilution Pulsiocath; Pulsion Medical Systems) inserted in either the contralateral carotid artery (pig) or the right femoral artery (baboon).

Hemodynamic evaluation was performed after completion of venous and arterial cannulations after restoring normovolemia during steady state. A minimum of three transpulmonary thermodilution measurements was taken with 5 or 10 mL iced sodium chloride 0.9% injections depending on body weight, in accordance with the manufacturer's recommendations. Data were recorded with PiCCOWin 6.0 software (Pulsion Medical Systems). For the modified hemodynamic treatment algorithm, 2.5% percentiles (CI, GEDI) and 97.5% percentile (ELWI) from the baboon group were used as cutoff values. Calculation of parameters derived from TPDT is presented in Table 1.

2.4 | Offline analysis and statistics

Recorded data were visualized with PiCCOWin 6.0 software. Low-quality measurements were excluded depending on the thermodilution curve, according to clinical standards. Data were then processed with Excel (Microsoft). To normalize the absolute values to body surface area (BSA), the formulas $0.0734 \times TBW^{0.656}$ (pigs), $0.078 \times TBW^{0.664}$ (female baboons), or $0.083 \times TBW^{0.639}$ (male baboons) were used, where TBW is total body weight in kg.[12,13] Data analysis was performed with GraphPad Prism 7.0 (GraphPad Software Inc). Where applicable, human reference values are presented for comparison.[14,15] Data are presented as mean ± standard deviation, median, and 95% interval. Hemodynamic measurements from pigs and baboons were compared using the Mann-Whitney rank sum test. Exact p-values are given for each test; for correlations, Pearson's r is indicated. Statistical significance was assumed when $P < .05$.

TABLE 1 Parameters assessed by transpulmonary thermodilution

Definitions		Calculation
CO	Cardiac output	$[(T_b - T_i) \times V_i \times K]$/AUC
SV	Stroke volume	CO/HR
SVR	Systemic vascular resistance	[(MAP − CVP)/ CO] × 80
ITTV	Intrathoracic thermal volume	CO × MTt
PTV	Pulmonary thermal volume	CO × DSt
GEDV	Global end-diastolic volume	ITTV-PTV
ITBV	Intrathoracic blood volume	1.25 × GEDV
EVLW	Extravascular lung water	ITTV-ITBV

Note: The following parameters were measured directly: T_b, blood temperature; T_i, injectate temperature; V_i, injectate volume; AUC, area under the thermodilution curve; HR, heart rate; MTt, mean transit time; DSt, exponential downslope time; MAP, mean arterial pressure; CVP, central venous pressure; K, correction constant (comprises specific weight and specific heat of blood and injectate fluid).

3 | RESULTS

Results from hemodynamic monitoring and TPTD measurements are summarized in Tables 2 and 3. Measurements from 47 baboons and 45 pigs were analyzed. Mean body weights of the two groups were 19.1 ± 5.2 kg and 17.3 ± 4.8 kg ($P = .0601$).

3.1 | Correlation of absolute parameters with body weight and body surface area

Figure 1 shows parameters of cardiac output (A, CO), afterload (B, SVR) and preload (C, GEDV), and pulmonary edema (D, EVLW), correlated with BSA or TBW, respectively. The overall correlation was significant; the correlation coefficient was higher in measurements from piglets compared with those from baboons (Pearson r as indicated in Figure 1; $P < .01$). SVR had a negative correlation coefficient ($r = -0.4435$ and $r = -0.5858$; $P < .01$), indicating decreasing resistances with increasing BSA.

3.2 | Hemodynamic monitoring

Table 2 shows the results from hemodynamic monitoring. MAP was 60% higher in baboons than piglets (Figure 2). Systolic (SAP, 49%) and diastolic arterial pressures (DAP, 68%) were also higher in baboons. Baboons had a 14% slower heart rate than pigs. Central venous pressure (CVP) was not significantly different between the two species.

3.3 | Transpulmonary thermodilution measurements

CI was 20% lower in baboons than pigs (Table 3, Figure 2). Since SVI was similar in both species, the increased CI must be due to an

TABLE 2 Results of hemodynamic monitoring in baboons (n = 47) and piglets (n = 45), presented as mean ± SD, median and 95% interval

	Baboon				Piglets			Human
	Mean ± SD	Median	95% interval	*P*	Mean ± SD	Median	95% interval	Reference
SAP (mm Hg)	124 ± 24	125	77-167	<.0001	83 ± 11	81	64-107	<120
DAP (mm Hg)	79 ± 20	84	43-113	<.0001	47 ± 12	44	30-78	<80
MAP (mm Hg)	99 ± 22	102	62-136	<.0001	62 ± 11	59	42-93	70-90
HR (bpm)	93 ± 13	92	71-126	.0006	108 ± 22	107	64-164	50-100
CVP (mm Hg)	8 ± 5	5	1-17	.2938	6 ± 3	6	1-15	2-6

Note: Statistical differences tested with the Mann-Whitney rank sum test ($P < .05$). Adult human reference ranges are shown for comparison.[14,15] SAP, systolic blood pressure; DAP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

TABLE 3 Indexed parameters derived from transpulmonary thermodilution measurements in baboons (n = 47) and piglets (n = 45), presented as mean ± SD, median and 95% interval

	Baboon				Piglets			Human
	Mean ± SD	Median	95% interval	*P*	Mean ± SD	Median	95% interval	Reference
CI (l/min/m^2)	4.2 ± 0.9	4.2	2.5-6.1	<.0001	5.3 ± 1.0	5.3	3.7-7.1	3.0-5.0
SVI (mL/m^2)	47 ± 15	44	24-93	.0674	51 ± 11	50	28-77	40-60
SVRI (dyn*s/cm^5*m^2)	1823 ± 581	1873	766-2986	<.0001	827 ± 204	805	372-1205	1700-2400
GEDI (mL/m^2)	523 ± 103	519	340-776	<.0001	433 ± 78	430	314-705	680-800
ELWI (mL/kg)	10 ± 3	9	5-15	.001	11 ± 2	11	8-18	3-7

Note: Statistical differences tested with the Mann-Whitney rank sum test ($P < .05$). Adult human reference ranges are shown for comparison.[14] CI, cardiac index; SVI, stroke volume index; SVRI, systemic vascular resistance index; GEDI, global end-diastolic volume index; ELWI, extravascular lung water index.

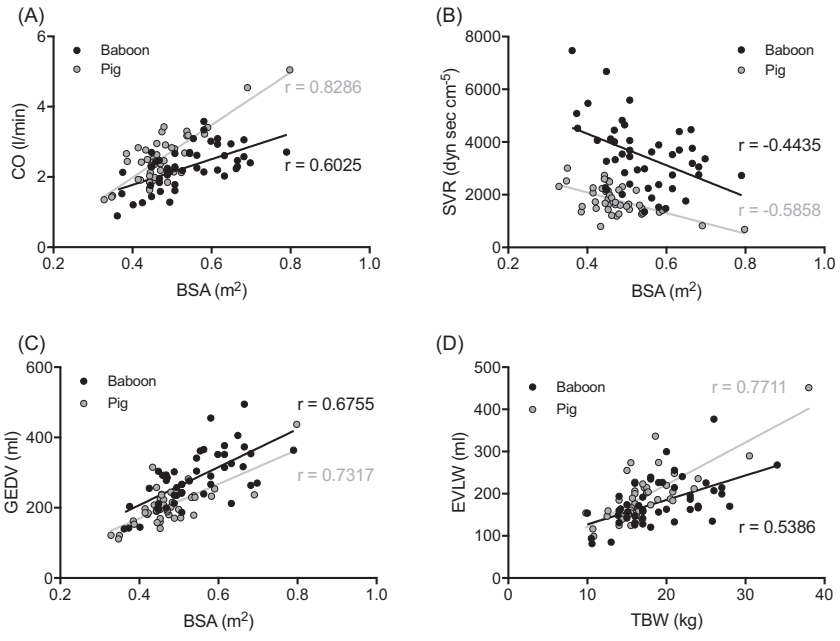

FIGURE 1 Correlations between body surface area (BSA) or total body weight (TBW) and parameters of transpulmonary thermodilution: A, cardiac output (CO); B, systemic vascular resistance (SVR); C, global end-diastolic volume (GEDV); D, extravascular lung volume (EVLW). Pearson r as indicated; $P < .01$

increased heart rate in piglets. SVRI was 120% higher in baboons than in pigs. The volumetric parameter of cardiac preload GEDI was 21% higher in baboons than pigs, whereas the extravascular lung water index (ELWI) was 15% lower.

3.4 | Comparison with human reference values

With the exception of CI and SVI (Table 2, Figure 3A), adult human reference values differed in all other parameters from the

measurements taken from baboons and piglets. MAP and HR of baboons were higher than human reference ranges (Table 2). In comparison, piglets had lower MAP and higher HR. SVRI measured in baboons was within the human reference range, but SVRI in piglets was approximately half that in humans and well below the reference range (Table 2, Figure 3B). The volumetric preload parameter GEDI was lower in both pigs and baboons than in humans (Table 3, Figure 3C), whereas the parameter of pulmonary edema (ELWI) was above human references values (Figure 3D).

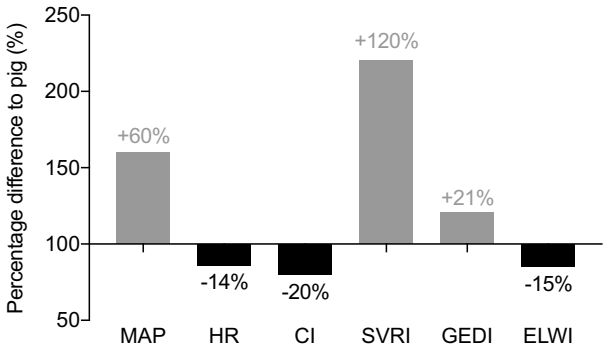

FIGURE 2 Percentage difference of hemodynamic and TPTD parameters of baboons with respect to piglets. Pig parameters are set to 100%. Mean arterial pressure (MAP), cardiac preload, and afterload (GEDI, SVRI) are higher in baboons than pigs, heart rate (HR), cardiac index (CI), and extravascular lung water index (ELWI) are lower. GEDI, global end-diastolic volume index; SVRI, systemic vascular resistance index

4 | DISCUSSION

4.1 | Cardiac output and afterload

Our study has revealed significant differences in the hemodynamic parameters of cardiac output and afterload of normovolemic, anesthetized, weight-matched baboons, and piglets. Cardiac output measurements from baboons and pigs correspond well to those from previous reports.[16-21] Differences in blood pressures have been described by authors working with juvenile pigs[19-22] and baboons.[16-18] Older studies also confirm the twofold difference in systemic vascular resistance between baboons[17,23,24] and pigs[19-22,25] with body sizes similar to the animals we examined.

Discussion of pigs as organ donors for humans has mainly focused on adult donors and recipients.[26,27] Full-grown pigs have cardiac output and arterial blood pressure values comparable to, or even higher than, adult humans.[26-28] Systemic vascular resistance—as well as pulmonary vascular resistance—in adult pigs has been reported as twice that in humans, supposedly giving the transplanted pig heart an advantage in pumping against lower resistance.[26,27]

In contrast, transplanting a juvenile porcine heart accustomed to low vascular resistance into an adolescent baboon with twice the resistance challenges the pig heart's ability to adapt to higher afterload. For most xenotransplantation experiments, clinically approved ischemic preservation is used for organ storage. In 40%-60% of these experiments, the grafts fail within 48 hours due to perioperative xenograft dysfunction (PCXD).[29] We hypothesize that ischemia/reperfusion injury caused by ischemic storage impairs the

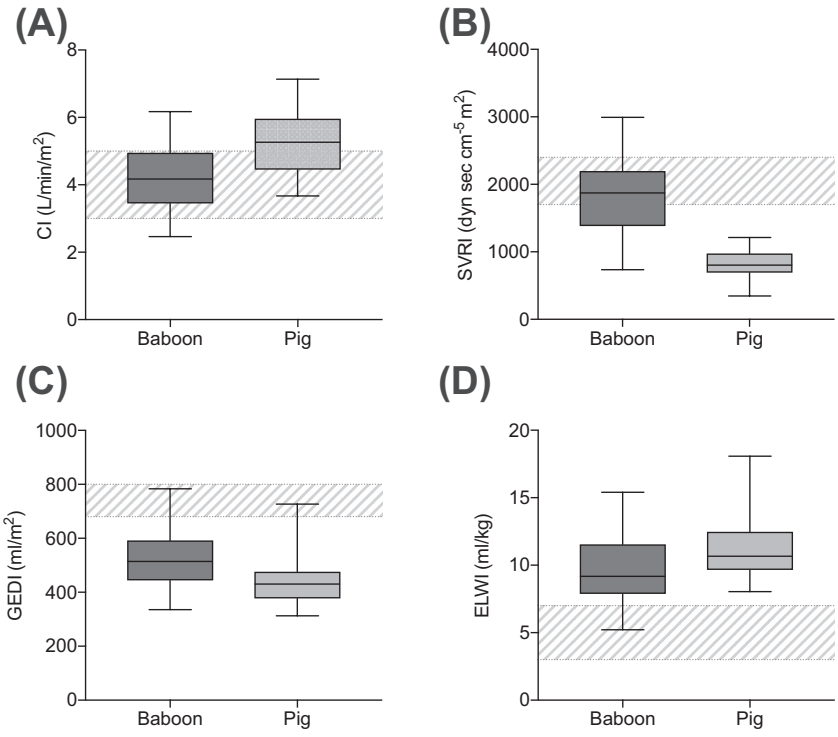

FIGURE 3 Transpulmonary thermodilution measurements in baboons and pigs and comparison with human reference ranges: A, cardiac index (CI); B, systemic vascular resistance index (SVRI); C, global end-diastolic volume index (GEDI); D, extravascular lung volume index (ELWI). Data are presented as box and whisker plots indicating mean, interquartile range, and minimum to maximum, striped areas represent adult human reference ranges[14]

ability of the pig heart to cope with increased systemic afterload, similar to acute right ventricular failure due to pulmonary hypertension in human allotransplantation. This could explain why PCXD is potentially reversible with time (recovery from ischemia/reperfusion injury)[29] and why PCXD does not occur when the porcine grafts are preserved by continuous non-ischemic organ perfusion (prevention of ischemia/reperfusion injury).[9]

Moreover, chronically elevated afterload triggers pressure overload-induced cardiac hypertrophy in humans patients.[30] When compensatory mechanisms fail, concentric myocardial hypertrophy eventually leads to diastolic pump failure. Recently, we observed a similar phenomenon in baboons that had undergone orthotopic xenotransplantation of porcine hearts.[9] Diastolic pump failure was prevented with a combination of anti-hypertensive and anti-proliferative treatments, indicating that chronically elevated afterload might, at least in part, explain excessive graft growth after cardiac xenotransplantation.

4.2 | Cardiac preload and extravascular lung water

For many years, perioperative volume therapy has been guided by central venous (CVP) and pulmonary artery occlusion pressures. However, these parameters do not accurately reflect cardiac preload.[31] TPTD provides the volumetric parameter global end-diastolic volume index (GEDI), which represents the sum of end-diastolic volumes of all four heart chambers. GEDI has been shown to be superior to filling pressures for guiding cardiac preload.[7] TPDT also provides the parameter ELWI, which reflects the fluid that is contained within the perfused regions of the lungs.[7] Elevated ELWI is typically found in pulmonary edema[6] and has been used as a therapeutic guide after cardiac surgery.[32]

To allow proper fluid management in xenotransplantation experiments, normal values are required for donor and recipient animals. The results from our baboon and piglet groups indicate that their normal GEDI values are much lower than reference values for adult human patients (680-800 mL/m^2). In contrast, ELWI is higher in both baboons and infant pigs than in adult humans (3-7 mL/kg). Low GEDI and high ELWI values have also been reported for other animals and human infants: In pediatric patients (n = 101 children, age 0-18 years), median GEDI was between 366 and 479 mL/m^2 and median ELWI between 10 and 12 mL/kg.[33-35] In Maryland minipigs (n = 38, 8-16 kg), López-Herce et al observed mean values of 198 mL/m^2 for GEDI and 16 mL/kg for ELWI, respectively.[36] ELWI values determined by gravimetry in newborn healthy lambs were more than twice as high as in adult sheep (13.3 vs 6.1 mL/kg).[37]

Lemson et al proposed that the lower GEDI values and higher ELWI values in younger children were a result of age-related changes in the ratio of lung weight to body weight and in the ratio of heart weight to BSA.[38] The greater lung weight in infants has consequences for calculating the (non-indexed) EVLW, for which the intrathoracic blood volume (ITBV) is needed. Historically, intrathoracic volumes were measured using the transpulmonary double-indicator (thermo-dye) dilution technique with two different indicators (cold and indocyanine green). Sakka et al empirically found ITBV, the sum of GEDV and pulmonary blood volume, to be ~1.25 × GEDV.[39] This linear relationship was incorporated into the single indicator methodology for TPTD. In children, this multiplier varies from 1.5 in the newborns to 1.2 in adults.[33] Therefore, application of the adult formula underestimates ITBV and overestimates EVLW. Similar to human infants, Rossi et al found ITBV to be 1.52 × GEDV + 49.7 (mL) for landrace piglets (24-32 kg), thus greatly improving the accuracy of estimating EVLW.[40] For baboons, the exact relationship is unknown.

These findings emphasize that human reference values of TPTD parameters for volumetric preload and lung water are age- and species-dependent and cannot be simply adopted in pig-to-baboon xenotransplantation. The normal values provided by this study may serve as a reference for both baboons and pigs, but we caution that comparisons should be restricted to animals of the same species and similar age and body size.

4.3 | TPTD parameters in xenotransplantation—modified hemodynamic decision model

TPTD provides data on many hemodynamic parameters. These require thoughtful interpretation and can be overwhelming for researchers unfamiliar with the method. To facilitate the use of TPTD for pig-to-baboon xenotransplantation experiments, we modified the manufacturer's hemodynamic treatment algorithm (PiCCO, Pulsion Medical Systems) with easily memorable values according to results from the baboon group. Figure 4 represents a simple theoretical decision model for a goal-directed therapy.

CI is the most important global parameter of circulatory function. Circulatory failure, indicated by a severe decrease in CI, results in insufficient peripheral oxygen delivery and must be treated promptly (Figure 4A). Treatment options are mainly volume therapy and catecholamines/vasoactive drugs.

- Low preload (GEDI < 350 mL/m^2) indicates decompensated hypovolemia and must be primarily treated with volume (crystalloid/colloid infusions, blood products in case of bleeding).
- Low preload and signs of pulmonary edema (ELWI > 15 mL/kg) are typical for inflammatory pulmonary disease with septic shock and not common in xenotransplantation experiments. Volume therapy should be applied cautiously and accompanied by catecholamine therapy.
- Low CI in the context of adequate preload (GEDI > 350 mL/m^2) after cardiac transplantation indicates left ventricular insufficiency and the need for catecholamine support (noradrenaline/adrenaline).
- Adequate preload and pulmonary edema (ELWI > 15 mL/kg) are signs of volume overload in severe left ventricular failure and must be treated with inotropic support and volume withdrawal (diuretics).

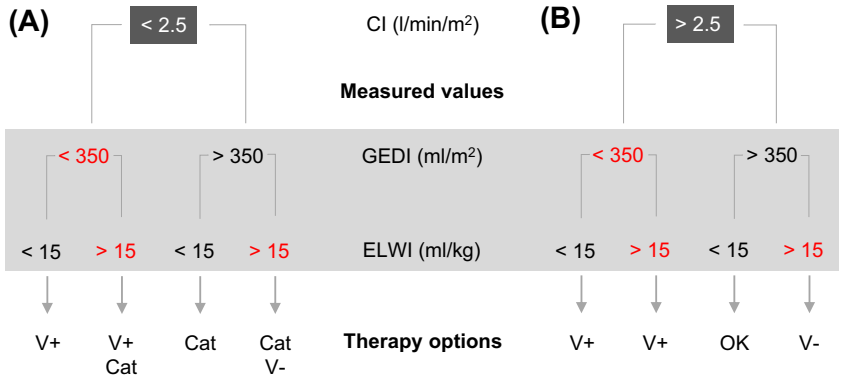

FIGURE 4 Hemodynamic decision model for cardiac pig-to-baboon xenotransplantation based on the manufacturer's recommendations for human adults (PiCCO, Pulsion Medical Systems), modified with normal values obtained from anesthetized baboons. See text for detailed explanations. CI, cardiac index; GEDI, global end-diastolic volume index; ELWI, extravascular lung water index; V+, volume loading; V–, volume withdrawal; Cat, catecholamines or vasoactive agents

Normal CI indicates a compensated circulatory function and does not usually require immediate therapeutic intervention. However, TPTD can help fine-tune or maintain an adequate drug therapy (Figure 4B).

- Low preload (GEDI < 350 mL/m^2) indicates compensated hypovolemia and must be primarily treated with volume (crystalloid/colloid infusions, blood products in the case of bleeding).
- Low preload and signs of pulmonary edema (ELWI > 15 mL/kg) are typical for inflammatory pulmonary disease and not common in xenotransplantation experiments. Volume therapy should be applied cautiously.
- Adequate preload (GEDI > 350 mL/m^2) and low ELWI are the aims of goal-directed therapy. Current volume and catecholamine therapy can be maintained.
- Adequate preload and pulmonary edema (ELWI > 15 mL/kg) are signs of compensated left ventricular insufficiency and should be treated with volume withdrawal.

TPDT measurements should be repeated regularly, especially after treatment changes or when hemodynamic parameters suddenly deteriorate. Circulatory insufficiency (CI < 2.5l/min/m^2) should always lead to a search for possible causes. TPDT can identify circulatory impairment and helps with therapeutic decision-making but cannot substitute for thorough investigation such as echocardiographic imaging. As for all hemodynamic monitoring, it is advisable to interpret absolute values with caution. This is especially so for ELWI, the calculation of which is based on a constant derived from adult human patients and cannot be directly translated to other species. Also, perioperative TPTD parameters should be compared with baseline measurements. A steady deterioration of one parameter, even if still within the reference range, usually indicates the need for intervention.

4.4 | Limitations

All measurements were taken under general anesthesia prior to surgical intervention. Anesthetics and analgesics, mainly propofol, iso/sevoflurane, and fentanyl, have various degrees of vasodilatory, negative inotropic, and negative chronotropic effects. As such, our findings do only apply to laboratory animals under general anesthesia and differ from alert animals. However, hemodynamic monitoring with TPTD is most useful during and shortly after surgery, when the animal is still anesthetized. Subsequent monitoring would be most desirable, but it is not feasible to have an intraarterial catheter in an awake animal.

Our study includes data from several experimental study protocols with different personnel performing anesthesia and surgery. Different anesthesia protocols seem to have only negligible effects on hemodynamic parameters. Bauer et al demonstrated that intravenous and inhalational anesthetics provide equal hemodynamic stability before surgery.[41] Although the choice and dosage of anesthetic (intravenous vs inhalational) did differ, pooling data from different study groups should provide reliable normal values.

Finally, we measured normal values for piglets in wild-type animals, while xenotransplantation experiments generally use genetically modified animals. In our experience, the typical genetic modifications (α1,3-galactosyltransferase knockout, human CD46, and human thrombomodulin) have no influence on hemodynamic parameters. Because of the scarcity of transgenic animals and their value for xenotransplantation experiments, we performed TPDT measurements in only a few animals with different genetic modifications (n = 8, data not shown). These revealed no significant differences as compared to wild-type animals, other than a slightly higher mean ELWI. All measurements were within the 95% interval presented in this study.

5 | SUMMARY

In summary, we present normal values for TPTD measurements in anesthetized baboons and piglets used for xenotransplantation experiments. There are important differences between these

animals with regard to systemic arterial pressure and vascular resistance, the latter being twice as high in baboons as in juvenile pigs. Elevated cardiac afterload may in part explain two phenomena that have been observed in the orthotopic pig-to-baboon cardiac xenotransplantation model: perioperative xenograft dysfunction and post-operative cardiac overgrowth. TPDT is a powerful tool to guide hemodynamic treatment after surgery, but human reference values and algorithms provided by the manufacturer should not be applied. For the purpose of pig-to-baboon cardiac xenotransplantation, we have modified the algorithm for a perioperative goal-directed therapy guideline.

DISCLOSURE

Mark Konrad is Head of Medical of Pulsion Medical Systems, Getinge.

AUTHOR CONTRIBUTIONS

Matthias Längin, Mark Konrad, Andreas Bauer, and Jan-Michael Abicht contributed to concept design, conducted experiments, data collection, data analysis, and drafted the article. Tanja Mayr, Stephanie Vandewiele, Johannes Postrach, Maren Mokelke, Julia Radan, and Paolo Brenner conducted experiments and data collection, contributed to data analysis, and approved the final draft. Bruno Reichart secured fundings, conducted experiments, and critically revised the article.

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Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival

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Xenotransplantation using pig organs has achieved survival times up to 195 days in pig orthotopic heart transplantation into baboons. Here we demonstrate that in addition to an improved immunosuppressive regimen, non-ischaemic preservation with continuous perfusion and control of post-transplantation growth of the transplant, prevention of transmission of the porcine cytomegalovirus (PCMV) plays an important role in achieving long survival times. For the first time we demonstrate that PCMV transmission in orthotopic pig heart xenotransplantation was associated with a reduced survival time of the transplant and increased levels of IL-6 and TNFα were found in the transplanted baboon. Furthermore, high levels of tPA-PAI-1 complexes were found, suggesting a complete loss of the pro-fibrinolytic properties of the endothelial cells. These data show that PCMV has an important impact on transplant survival and call for elimination of PCMV from donor pigs.

Recently, consistent success in life-supporting (orthotopic) porcine cardiac xenotransplantation has been reported[1]. In that study hearts from α1,3-galactosyltransferase-knockout (GTKO) pigs that express human membrane cofactor protein (CD46) and human thrombomodulin (hTM) had been transplanted into baboons and survival times up to 195 days were achieved. This is a milestone on the way to clinical cardiac xenotransplantation which is urgently needed: The supply of human organs does not match the needs and many patients with terminal cardiac failure die while being on the waiting list.

Xenotransplantation with genetically modified porcine organs, as an alternative to allogenic (human-to-human) procedures may be associated with the transmission of porcine microorganisms, among them the porcine endogenous retroviruses (PERVs). PERVs are integrated in the genome of all pigs and they are able to infect human cells[2]. However, until now no PERV transmission was observed in the first preclinical (for review see ref.[2]) and clinical xenotransplantation trials[3,4]. Whereas PERV-A and PERV-B, which are present in all pigs, infect human cells, PERV-C infects only pig cells and is not present in all pigs[2]. However, recombinations between PERV-A and PERV-C can occur and the recombinant PERV-A/C is characterised by a higher replication competence[5]. Therefore, it is highly recommended to use PERV-C-free pigs for xenotransplantation. Interestingly, using CRISPR/Cas all retroviral sequences can be inactivated in the pig genome[7], however, it is still unclear whether this is needed for a safe xenotransplantation[9].

From the other viruses widely distributed in pigs, the porcine cytomegalovirus (PCMV) is of great concern[10]. PCMV is related with the human cytomegalovirus (HCMV), also called human herpesvirus 5 (HHV-5). HCMV causes fatal infections in human organ transplant recipients if not treated, leading to end-organ disease, such as gastrointestinal ulceration, hepatitis, pneumonitis or retinitis. HCMV can also lead to systemic infection and disease once a threshold value of virus load is exceeded[11]. In fact, HCMV has also been detected in the bowel mucosa where its reactivation has been suggested to lead to IL-6 release and inflammatory bowel disease[12].

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Animal number	ID number	Group[a]	PCMV in the explanted heart (copies per reaction)	Survival time (days)
A (1)[b]	16,752	I	368	3
B (2)	16,754	I	30	1
C (3)	16,755	I	1.940.000[c]	30
D (4)	16,751	I	32	1
E (5)	16,753	I	62	1
F (6)	16,048	II	839.000	18
G (7)	17,138	II	0[d]	4
H (8)	17,140	II	39.200	27
I (9)	17,139	II	2.950.000[e]	40
P	17,494	III	33.000.000	15
Q	17,492	III	15.350.99	27

Table 1. PCMV infection in explanted pig hearts from different baboons. [a]Treatment groups according to Längin et al. 2019. [b]In brackets animal number as in Längin et al. 2019[1]. [c]PCMV was also detected in the blood and the serum of baboon C (3×10^4 genome equivalent/ml blood) (Morozov et al., 2016, baboon 57)[26], [d]No PCMV was found in different organs of baboon G (Fiebig et al. 2018, baboon 62)[27], [e]PCMV was found in the spleen, liver, kidney, blood of baboon I (between 100 and 10,000 copies / 100 ng DNA) (Fiebig et al., 2018, baboon 64)[27].

Meanwhile it was shown that PCMV is a roseolovirus and more closely related with human herpesviruses 6A, 6B and 7 (HHV-6A, HHV-6B, and HHV-7)[13]. Therefore the thermology PCMV is to a certain degree misleading and should actually be porcine roseolovirus (PCMV/PRV)[14]. The International Committee on Taxonomy of Viruses (ICTV) classified this virus as suid betaherpesvirus 2[15]. The closely related HHV-6 and HHV-7 were reported to be associated with numerous diseases, e.g., liver failure[16], multiple sclerosis[17] and Alzheimer disease[18]. Furthermore, HHV-6 was found to promote cancer development[19] and accelerate acquired immunodeficiency syndrome (AIDS) in humans and monkeys[20,21], possibly by its immunosuppressive property[21–25].

Recently, by analysing a baboon recipient of orthotopic pig heart transplantation with a relatively short survival time (29 days) and hepatic failure, PCMV/PRV infection was observed in the recipient[26]. In addition, PCMV/PRV transmission was also found in two other baboon recipients with 4 and 40 days of transplant survival. Immunohistochemical studies of the recipient baboons showed PCMV/PRV-expressing cells in all organs of the animal, most likely representing disseminated pig cells[27]. These data, together with similar data on pig kidney xenotransplantions in non-human primates[28,29], suggest that PCMV/PRV significantly reduces the survival of pig xenotransplants. However, the mechanism through which PCMV/PRV reduces transplant survival is still unclear[30].

To better understand the impact of PCMV/PRV on pig transplant survival in orthotopic heart transplantation, numerous donor pig—baboon recipient pairs were retrospectively analysed for PCMV/PRV transmission. Here, we demonstrate for the first time that PCMV transmission in orthotopic pig heart xenotransplantation is associated with a reduced survival time of the transplant and indicate an impact of PCMV/PVR on cytokine release and coagulation. Furthermore, we show that other porcine viruses, which could potentially impact xenotransplant survival, including PERVs, hepatitis E virus (HEV), three porcine lymphotropic herpesviruses (PLHV) and the porcine circoviruses (PCV) 1 and 2, had not been transmitted.

Results

Transmission of PCMV/PRV into baboons after orthotopic pig heart transplantation. All baboon recipients received an immunosuppression including an induction therapy with an anti-CD20 antibody, anti-thymocyte-globulin and a monkey-specific anti-CD40 monoclonal antibody or humanized anti-CD40LPA-Sylated as described in detail[1]. Three groups of animals have been transplanted. In group I, donor organs were preserved with two clinically approved crystalloid solutions, the animals survived for less than 30 days (animal C, Table 1) and suffered from perioperative cardiac xenograft dysfunction (PCXD). To reduce the PCXD, in group II the pig hearts were preserved with an oxygenated albumin-containing hyperoncotic cardioplegic solution. Three of the four animals of this group lived for 18 (animal F), 27 (animal H) and 40 (animal I) days. A diastolic heart failure and subsequent congestive liver damage resulting from massive cardiac overgrow were observed in these animals. To prevent this, in group III baboon recipients were weaned from cortisone at an early stage and received antihypertensive treatment since pigs have a lower systolic blood pressure than baboons. In addition, a temsirolimus medication was used to counteract cardiac overgrowth. Two recipients in this group survived for 195 (animal O, Table 2) and 182 (animal N) days[1].

When the recipient baboon organs from group I and group II were analysed for PCMV/PRV transmission, PCMV/PRV was detected not only in the explanted pig heart but also in several different organs from all baboons except one (G; Table 1). Some of these animals (B, D, E) survived only one day and showed very low virus load in the explanted heart. However, after only three days an increase of the virus load was observed (animal A). When analysing the animals with the longer survival time, e.g., animal F with 18 days, animal H with 27 days, animal C with 30 days and animal I with 40 days, it became evident that PCMV/PRV replicates over time. Animal I

Animal	ID number	Transplant survival days	PCMV real-time PCR	BaCMV real-time PCR	HEV		PLHV1, 2 PCR	PLHV3 PCR	PERV			PCV1,2 PCR
					ELISA/WB*	Real-time PCR			PERV PCR	PERV-C PCR	WB*	
Pig 5528			–		–	–	++	–	++	+		–
Baboon J	17,186	90	–	+	n.t	–	–	–	+**	–	–	n.t
Pig 5415			–	–	+	–	+++	–	++	+		–
Baboon K	17,187	50	–	+	–	–	–	–	+**	–	–	n.t
Pig 5420			–	–	+	–	+++	–	++	+		–
Baboon L	17,290	90	–	+	–	–	–	–	+**	–	–	n.t
Pig 5623			++		–	–	+	–	++	+		–
Baboon M	17,188	10	++	+++	n.t	–	–	–	+**	–	–	–
Pig 5803			–		–	–	+++	–	++	+		–
Baboon O	17,493	195	–	+	–	–	–	–	+**	–	–	
Pig 5807			–		–	–	+++	–	++	+		–
Baboon N	17,491	182	–	+	–	–	–	–	+**	–	–	–
Pig 6249			++		–	–	+++	–	++	+		–
Baboon P	17,494	15	+++	+	–	–	–	–	+**	–	–	–
Pig 6253			+		–	–	++	–	++	+		–
Baboon Q	17,492	27	++	+	–	–	–	–	+**	–	–	–

Table 2. Testing donor pigs and recipient baboons of transplantation group III for different viruses. *WB, Western blot assay; **indicating microchimerism; n.t., not tested.

(number 64 in publication 27) had not only a very high virus load in the explanted heart, but a high virus load was also found in the spleen, kidney, and blood (between 100 and 1000 copies/100 ng DNA) and cells expressing PCMV/PRV proteins were found in all organs of the transplanted animal[27]. The longer the transplant was present in the recipient the higher was the virus load in the explanted and formalin/ethanol fixed pig heart (Table 1). This rule is operative only to a certain threshold of the virus load, above this threshold the survival time decreases due to the pathogenic effect of the virus.

When the recipient baboons of group III (early cortisone tapering, antihypertensive treatment and temsirolimus) and their donor pigs were analysed, PCMV/PRV was found to be absent in the donor pigs and consequently in the transplanted baboons of animals with the longest survival times such as baboon J (90 days), baboon K (50 days), baboon L (90 days), baboon N (182 days) and baboon O (195 days) (Table 2). In contrast, PCMV/PRV was found in the donor pigs of transplanted baboons that showed a shorter survival rate like baboon P (15 days), baboon Q (27 days) and M (euthanized 10 days after transplantation because of severe iatrogenic complication) (Table 2). In the hearts explanted from animals P and Q astonishingly high copy numbers of PCMV/PRV were detected. Since all these data were obtained testing the formalin/ethanol fixed pig hearts, in addition the virus load in the frozen right ventricle of the heart explanted from baboon P was analysed and 48 million copies were found (33 million in the fixed heart). This is in the same magnitude, indicating that testing of fixed and frozen materials give approximately the same result.

Distribution of PCMV/PRV in pig and baboon organs. Using real-time PCR, the copy number (viral load) of PCMV/PRV was analysed in PBMCs and different organs of all donor pigs and in the organs of the transplanted baboons after removal of the transplanted heart, which was also analysed. As an example we illustrate in Fig. 1 the pair donor pig 6249 and the recipient baboon P. All other animals analysed had a similar pattern. The virus load in several organs of baboon I has been shown previously (animal 64 in reference 27). With exception of the skin, the viral load was high in all baboon organs (spleen, liver, muscle, kidney lung and lymph nodes) and donor pig organs (lung, spleen, liver, kidney and PBMCs), in agreement with the results in baboon I[27]. The highest viral load was found in the explanted pig heart (Fig. 1). This is the main place of virus replication and this replication is possible due to the absence of the pig immune system, which could have reacted against the virus in the donor pig, and due to the strong immunosuppression in the baboon inhibiting its immune system.

Pathological findings and modulation of cytokine release. As mentioned above, baboons N and O from treatment group III, showed the longest survival time, 182 and 195 days, respectively, compared to baboons P and Q, which survived only 15 and 27 days. Interestingly, both baboons P and Q were characterized by pathological and inflammatory changes, some of which might be the result of a PCMV/PRV infection, bearing in mind the broad disease spectrum induced by herpesviruses. Both animals showed signs of low-output heart failure at the end of the experiments, resulting in multi organ dysfunction as indicated by the pathological increase in functional parameters of liver, pancreas and kidney such as aspartate aminotransferase, creatine kinase, and lactate dehydrogenase. Decreased abdominal perfusion probably caused loss of mucosal barrier function in the gut, leading to translocation of intestinal microbes and to a strong increase of levels of interleukin-6 (IL-6) (Fig. 2). In fact, Klebsiella pneumonia was found in blood cultures of baboon Q one day before euthanasia. The

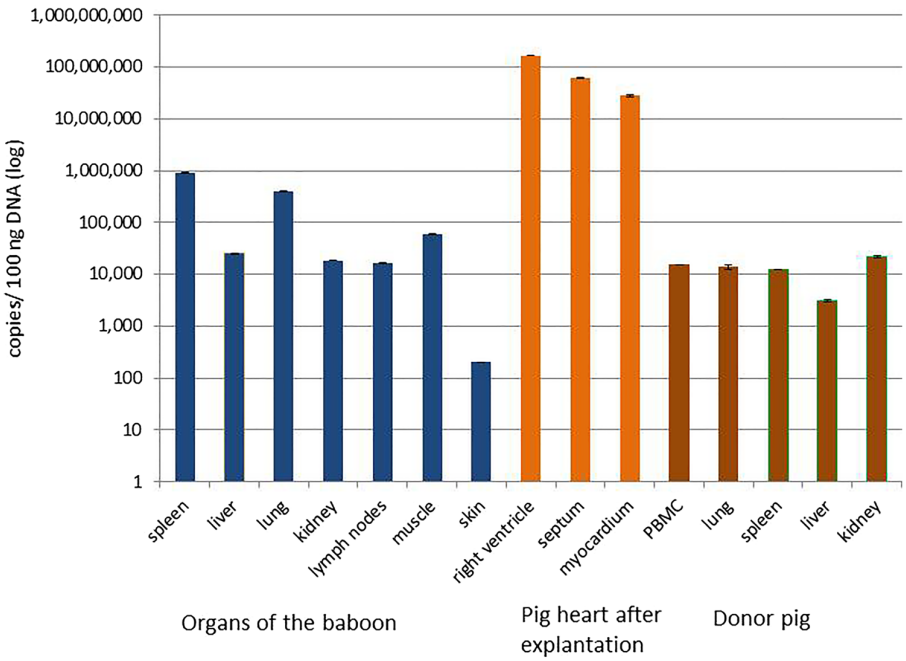

Figure 1. Viral load (copies per 100 ng DNA) in different organs of baboon P after explantation of the pig heart, in PBMCs and different organs of the donor pig and in the pig heart after explantation as measured by real-time PCR.

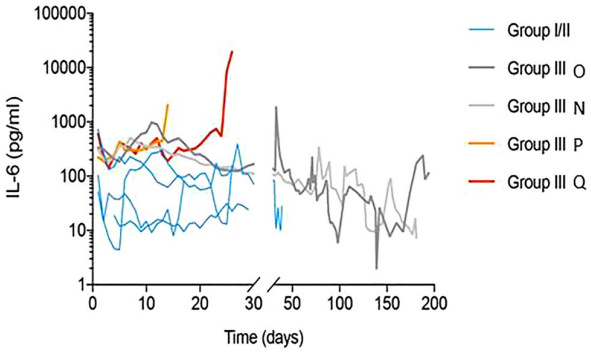

Figure 2. Kinetics of the IL-6 amount in the blood of transplanted baboons as measured by ELISA. The baboon recipients of the treatment group III are indicated (N, O, P, Q).

increase in IL-6 most probably is not due to treatment with an IL-6-receptor antagonist tocilizumab, since treatment began at the very beginning of transplantation and was given to all animals as part of the treatment protocol, including animals N and O that did not show high plasma levels of the cytokine. To confirm these data and to perform a broader analysis of further pro- and anti-inflammatory cytokines, a cytometric bead array (CBA) assay for non-human primate Th1/Th2 cytokines as well as an IL-10 ELISA were performed. The obtained data confirmed the strong increase of IL-6 (Fig. 3A), and also revealed a substantial increase of tumour necrosis factor (TNF) in baboons P and Q with the PCMV/PRV-positive hearts (Fig. 3B). No alterations were observed in serum levels of the proinflammatory cytokines interferon γ (IFN γ) (Fig. 3C) and interleukin 2 (IL-2) (Fig. 3D) as well as the anti-inflammatory cytokines interleukin-4 (IL-4) (Fig. 3E), interleukin-5 (IL-5) (Fig. 3F), and interleukin-10 (IL-10) (data not shown).

Interestingly, histological examination of the explanted pig hearts from baboons N, O, P, and Q revealed marked perivascular and moderate edema in otherwise unremarkable myocardial tissue (Fig. 4A, B). In particular, no morphologic signs of cellular or antibody-mediated transplant rejection was found (Fig. 4B), and in parallel no elevated levels of non-galactose-a1,3-galactose reactive IgM and IgG were measured (not shown), indicating that immunological rejection was not the cause of the reduced survival observed in animals P and Q.

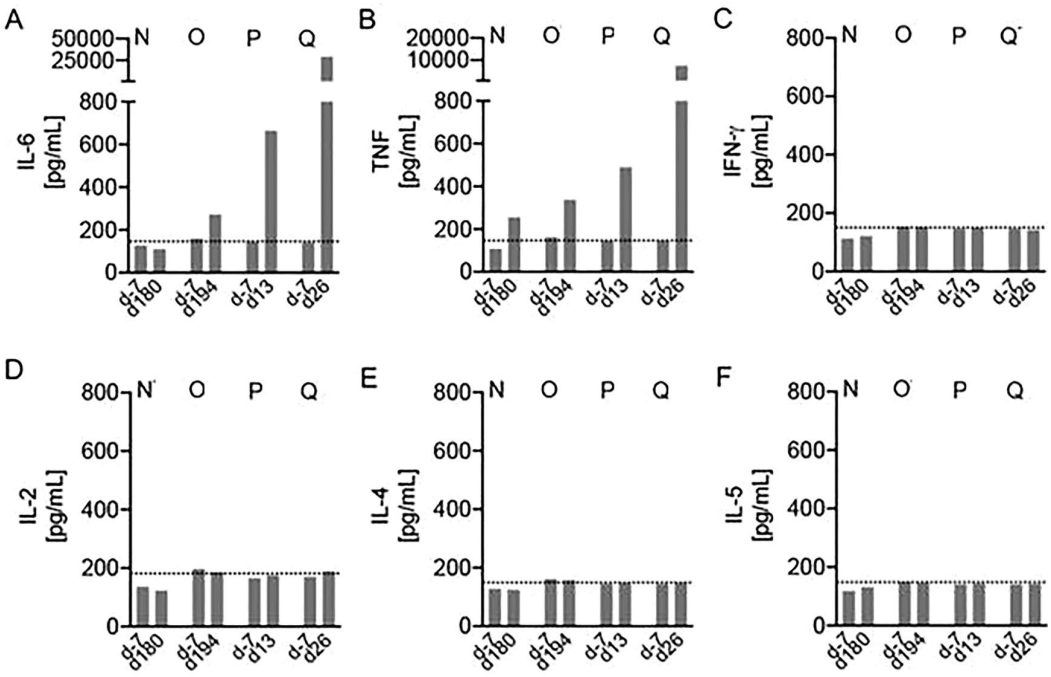

Figure 3. Increased IL-6 and TNF serum levels in baboons with PCMV/PRV-positive hearts. BD CBA Non-human primate Th1/Th2 kit was used to measure plasma levels of (**A**) IL-6, (**B**) TNF, (**C**) IFN γ, (**D**) IL-2, (**E**) IL-4 and (**F**) IL-5 one week before transplantation (day -7) and during the experiment (day 13, 26, 180 and 194, respectively) in baboons N, O, P and Q. Dotted lines indicate lowest standard concentration.

High levels of tPA-PAI-1 complex. To analyse whether animals infected with PCMV/PRV could present alteration in the coagulation system, tissue plasminogen activator (tPA) and plasminogen activator inhibitor 1 complexes (tPA-PAI-1) were measured in plasma samples by an ELISA[31]. Interestingly, we observed that baboons P and Q, which presented clear pathological alterations, also had very high levels of tPA-PAI-1 complexes (Fig. 5). This indicates a hypercoagulable state of the two animals and a decrease in fibrinolysis, originated most probably by endothelial cell damage.

Expression of BaCMV afterPCMV/PRV transmission. Since all baboon recipients carried BaCMV, we looked into a possible activation of this virus, which may also influence the survival time of the transplant and recipient. Recently a significantly increased expression of BaCMV in plasma of baboon I after transplantation of a PCMV/PRV-positive pig heart was measured[27]. Although analysis of BaCMV in all baboon organs (liver, kidney, spleen and lung) showed a high virus load, a clear and measurable increase of the BaCMV virus load in the blood after transplantation as was found in animal I was not observed (Table 2). Moreover, no differences in the BaCMV viral load in baboons with a long or a short survival time was observed. This highly suggests that activation of BaCMV is not the cause of early loss of the transplanted pig heart.

Prevalence and transmission of other porcine viruses. In order to analyse whether other porcine viruses were present in the donor pig and were transmitted to the recipient, which also may influence the survival time of the xenotransplant, both Western blot and PCR/RT-PCR analysis was performed to detect PERV, PLHV, PCV and HEV in both pig and baboon tissues. PLHV-1 and PLHV-2 had been detected in all donor pigs used in this study. Despite this, no transmission of these viruses to the recipient baboons was observed (Table 2). Although Western blot analyses showed HEV infection in the donor pigs of baboons G, I, K and L, detection of HEV by RT-PCR gave negative results in all donor pigs, indicating that the virus was not present in their blood. However, transmission to the recipients was not observed (Table 2). Indeed, all baboons were negative for HEV infection independently to the test used for analysis, e.g., no positive RT-PCR reactions and no positive ELISA or Western blot analyses were found.

PERV was detected as expected in all donor pigs by PCR using specific primers binding to a highly conserved region in the polymerase (pol) gene (Table 2). In fact, these primers recognise both PERV-A, PERV-B and PERV-C. Since PERV-A and PERV-B is present in all pigs[2], a PCR using primers specific for the env region of PERV-C was performed, and PERV-C was detected in all donor pigs.

PERV was also analysed in baboon samples using the pol specific PCR, detecting PERV-A, PERV-B and PERV-C. As shown in Table 2, these sequences were detected in all baboon blood samples, however the detection of PERV in the blood samples might be due to circulating cellular DNA from dead transplant cells or from circulating pig cells, a phenomenon called microchimerism. This was confirmed by the finding of porcine

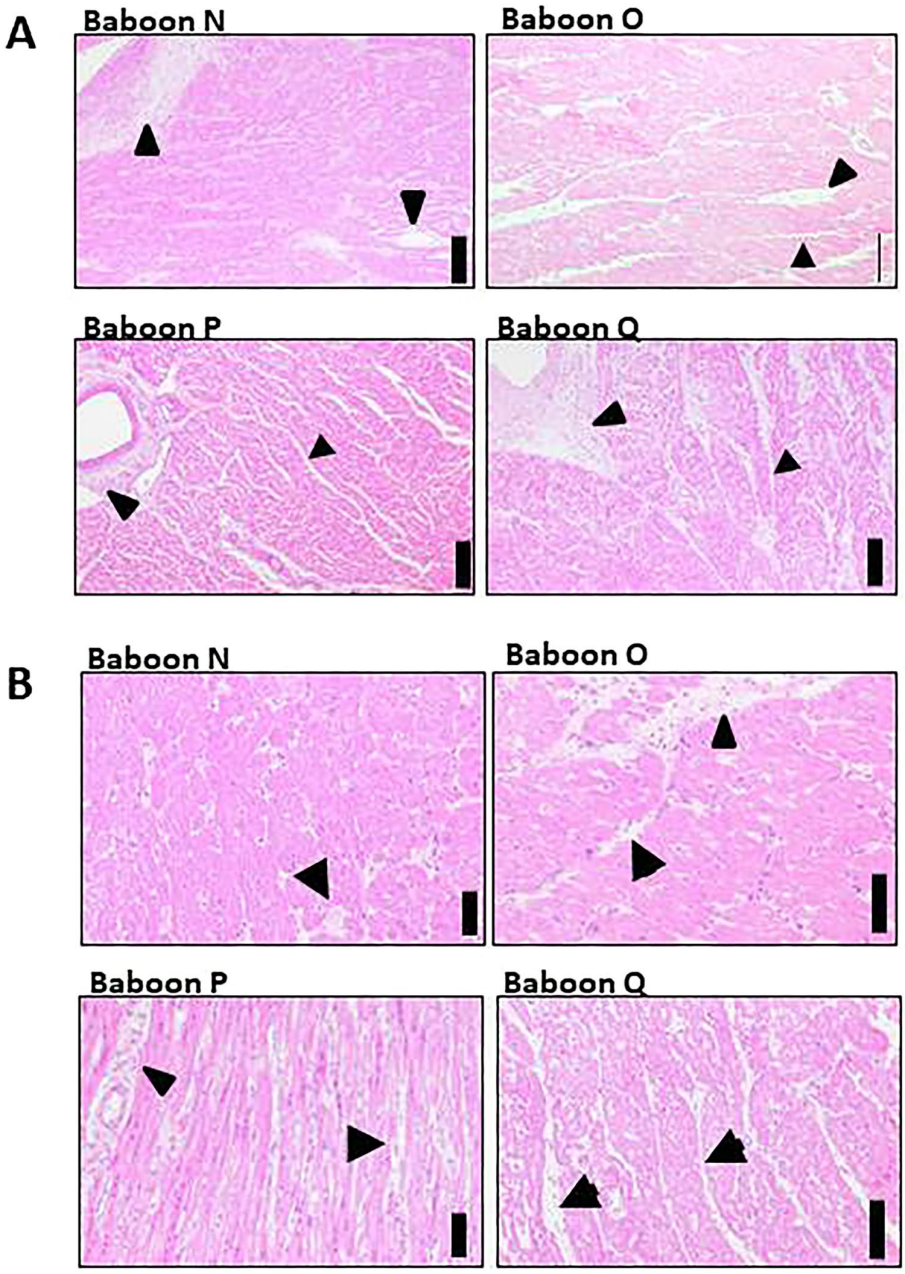

Figure 4. **A** Histopathologic evaluation of the explanted pig hearts from baboons N to Q. Arrowheads mark perivascular and arrows moderate edema in otherwise unremarkable myocardial tissue in all specimens. Scale bars indicate 250 µm. **B**. Histopathologic evaluation revealed perivascular (arrowhead) and interstitial (arrow) edema in otherwise unremarkable myocardial tissue in all specimens. In particular, no morphologic signs of cellular or antibody-mediated transplant reaction was found. Scale bars indicate 100 µm.

glycerinaldehyde-3-phosphat-dehydrogenase (GAPDH) by a specific PCR. Since the copy number of PERV-C in pig cells is much lower compared to the copy number of PERV-A and PERV-B, PERV-C sequences could not be detected in the blood of the baboons. Moreover, Western blot analysis showed no anti-PERV antibodies in all baboon samples, clearly indicating absence of infection (Supplementary Fig. 1). In summary, no other pig viruses which could influence the survival time of the transplant had been transmitted to the recipient baboons.

Discussion. Recently, survival times of 182 and 195 days after preclinical orthotopic pig-to-baboon xenotransplantation were achieved by several measures that include an improved immunosuppressive regi-

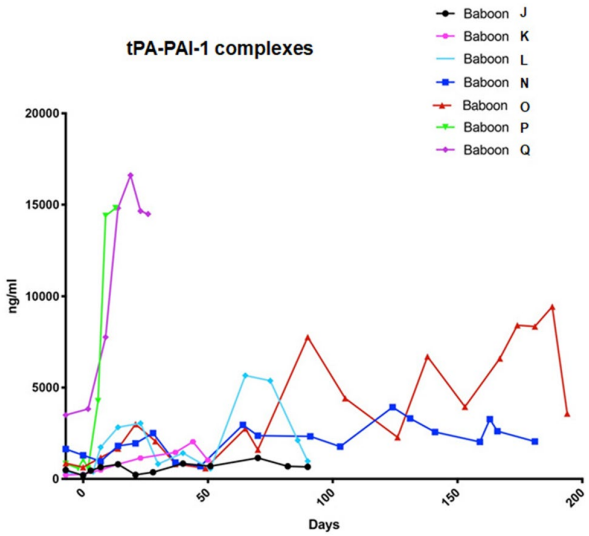

Figure 5. Measurement of tPA-PAI-1 complexes in the blood of baboons J65-Q74.

men, non-ischaemic preservation with continuous perfusion and control of post-transplantation growth of the transplant[1]. Here, for the first time, we show that the use of organs from PCMV/PRV-positive pigs in this life-supporting model is associated with a reduction of the transplant survival time.

Our data on reduction of the survival time of PCMV/PRV-positive pig hearts orthotopically transplanted into baboons supports previous findings in other preclinical trials on kidney xenotransplantations[28,29,32–36]. In these studies, PCMV/PRV was found in all organs of the baboon recipients, with the highest copy numbers in liver, lung and kidney[33]. An increased PCMV/PRV virus load was found in 22 different pig xenotransplants with a survival time between 7 and 32 days in baboons[33]. The presence of PCMV/PRV was usually found associated with consumptive coagulopathy (CC)[34]. However, there was no correlation between PCMV/PRV infection and CC, but lower levels of PCMV/PRV infection were always associated with a prolonged transplant survival[34]. When a heterotopic pig heart transplantation was performed in baboons, the survival time of the transplant was shorter when the organs were PCMV/PRV-positive (median 20 days), compared with PCMV/PRV-negative transplants (median 53 days)[35]. Most importantly, early weaning was shown to prevent PCMV/PRV infection of the pigs[35]. Organs from PCMV/PRV-free animals due to early weaning did not induce CC. Unfortunately, PCMV/PRV had been shown to have a reduced susceptibility to ganciclovir which is very effective in inhibiting HCMV[36]. Ganciclovir was also given to the baboons in the present study. When kidneys from GTKO pigs were transplanted into baboons, the median survival time of the kidneys from PCMV/PRV-positive animals was 14.1 days, the survival time of kidneys from PCMV/PRV-negative animals was 48.8 days and that of kidneys from PCMV/PRV-negative animals after a Caesarean delivery was 53 days[28], clearly demonstrating that the transmission of PCMV/PRV reduced significantly the kidney transplant survival. A similar effect was observed, when GTKO pig kidneys were transplanted into cynomolgus monkeys. Whereas the survival time of kidneys from PCMV/PRV-free animals was 28.7 days, the presence of PCMV/PRV reduced the survival time to 9.2 days[29].

The immunosuppression used in this trial is based on an induction therapy with an anti-CD20 antibody, anti-thymocyte-globulin and a monkey-specific anti-CD40 monoclonal antibody or humanized anti-CD40LPA-Sylated. During maintenance therapy methylprednisolone was reduced gradually, whereas mycophenolate mofetil and anti-CD40 monoclonal antibody or anti-CD40L PASylated Fab treatment remained constant as described in detail[1]. This strategy has been used successfully in other laboratories[2] and it was approved by the local Upper Bavarian Government. The selected immunosuppressive regimen will be easily translated into a future pig-to-human system. There are of course other immunosuppressive agents such as blocking leukocyte costimulatory molecules[37], expression of CTLA4-Ig, which disrupts co-stimulatory pathways, and PD-L1, which activates T-cell inhibitory pathway[38], and co-receptor blockage targeting CD4 and CD8[39], however these methods have not been applied in solid organ xenotransplantation, but mainly to prevent rejection of embryonic stem cells.

Our findings that PCMV/PRV induces IL-6 and TNF release and increases the amount of t PAI-1/tPA complexes significantly contributes to the understanding of the mechanism of action of PCMV/PRV on pig transplant survival. However, there are still open questions. It is still unclear, whether PCMV/PRV can infect baboon cells. Using PCR and immunohistochemical methods, PCMV/PRV was found in all organs of the transplanted baboon (Fig. 1 and reference 27), however it is unclear whether the cells stained positive with a specific antiserum against PCMV/PRV are disseminated PCMV/PRV-producing pig cells or infected baboon cells. It is also unclear whether PCMV/PRV can infect human cells. One report claimed infection of human cells[40], whereas another report showed that human cells cannot be infected with PCMV/PRV[41]. In our preliminary experiments incubating PBMCs from PCMV-positive pigs with human 293 cells, no infection of the human cells was observed (unpublished data). Since different non-human primate species (cynomolgus monkeys, baboons) showed a

similar reduction of the survival time (ref. 28, 29 and this study), it seems very likely that the same may happen in humans. It is important to note, that the high virus load in the recipient baboon, independent whether the virus was produced by the pig transplant or by infected baboon cells, caused rejection of the transplant mainly by acting on the cytokine production and coagulation and this cannot be analysed in a human system in vitro.

There are other factors which may contribute to the reduction of pig transplant survival time. A consumptive coagulopathy was often observed in non-human primates with pig xenotransplants[34,42–44]. Although in vitro an activation of the porcine tissue factor (TF) in porcine aortic endothelial cells by a PCMV/PRV infection was observed, no correlation between TF expression and PCMV/PRV infection was observed in vivo[34]. An enhanced expression of ICAM-1 (intracellular adhesion molecule I) and MHC (major histocompatibility complex) class II in the pig transplant in baboons suggests an activation of endothelial cells[28]. PCMV/PRV is like many other viruses immunosuppressive and was shown to modulate the expression of immune-related genes in pig immune cells[22]. Furthermore, a PCMV/PRV infection of pigs is usually associated with opportunistic bacterial infections on the basis of the suppressed immune system[23] and a transcriptome analysis of PCMV/PRV-infected thymuses showed an up- and downregulation of immune-regulatory genes[24]. However, considering the strong pharmaceutical immunosuppression of the animals in order to prevent rejection of the pig organ, the immunosuppressive properties of PCMV/PRV may not be important for the PCMV/PRV-induced pathogenesis.

An upregulation of IL-6 and TNFα in several allo- and xenotransplantation studies has previously been described indicating pathological roles for these cytokines (for review see[45]). IL-6 is one of the most important cytokines during an infection, along with IL-1 and TNF[46]. TNF is a cytokine involved in systemic inflammation and is a critical effector molecule in the immune response to viral pathogens[47]. TNF is able to inhibit viral replication and respond to sepsis via IL-1- and IL-6-producing cells.

The pleiotropic effects of IL-6 include the stimulation of antibody production and induction of acute-phase proteins, such as C-reactive protein (C-RP) and fibrinogen[48]. In a pig-to-baboon xenotransplantation study in which kidneys, hearts and artery patches from GTKO or GTKO/CD46-transgenic pigs were transplanted, significantly increased IL-6 levels were observed[49]. High levels of fibrinogen and C-RP positively correlated with IL-6 levels and C-RP depositions in the heart and kidney xenotransplants were detected. These data indicate that the up-regulation of IL-6 might promote systemic inflammation and together with the subsequent induction of C-RP leads to a dysregulation of coagulation after xenotransplantation[49]. Some studies suggest that the administration of tocilizumab, an antibody raised against the IL-6 receptor, reduced antibody-mediated rejection in post-transplantation and improved allograft survival[50] or reduced C-RP levels and other factors to alleviate consumptive coagulopathy[51–54]. Tocilizumab reacts with the human and baboon IL-6 receptor, but not with the pig IL-6 receptor[54]. Blocking the baboon IL-6 receptor may lead to higher circulating levels of IL-6n in the blood and this may be detrimental to pig xenotransplant survival due to IL-6 stimulation of pig cells in the transplant.

Additionally, in an in vitro model in which porcine aortic endothelial cells or PBMCs were incubated with human umbilical vein endothelial cells (HUVECS) an upregulation of human inflammatory cytokines including TNFα, chemokines and tissue factors by porcine IL-6 and TNFα was observed. These data indicate that porcine IL-6 and TNFα can stimulate recipient cells and thereby promote coagulation as well as inflammation upon xenotransplantation[55]. Besides IL-6 also TNFα was reported to be up-regulated in baboons after pig heart transplantation and seems to act as a key pro-inflammatory cytokine in xenotransplant rejection[56,57]. TNFα was also detected during acute rejection in porcine islets transplanted into non-human primates NHPs[58]. Furthermore, the TNFα antagonist etanercept was shown to suppress coagulation dysfunction in xenotransplantation and to contribute to pig kidney survival in baboons[59].

Cytokines released by immune cells can also directly influence the proliferation cardiomyocytes. Whereas murine Treg cells promote proliferation of murine neonatal cardiomyocytes through paracrine factors including cytokines[80], TNFα, IFNα and IL-17A produced by $CD4^+$ Th1 and Th17 cells reduced the number of cultured murine cardiomyocytes[61]. Therefore these cells directly inhibited proliferation and promoted the apoptosis of neonatal mouse cardiomyocytes.

Despite the seemingly overwhelming evidence for the role of PCMV/PRV, there may also be other factors contributing to the reduced survival rates. As explained elsewhere in detail[1], xenotransplantation experiments in groups I and II failed because of insufficient organ preservation and overgrowth of the transplant, respectively. Baboons P and Q (group III) were euthanized because of beginning multi-organ failure, which might be attributed to other causes than PCMV/PRV: Baboon P had received a graft of borderline small size and baboon Q developed recurrent pericardial effusions. Relative size mismatch may lead to systolic heart failure, whereas recurrent effusions may lead to pericardial tamponade and diastolic insufficiency, both eventually causing multi-organ failure. Microbial translocation and opportunistic infections were observed in animals P and Q; both are common after infection with immunosuppressive viruses including human immunodeficiency virus (HIV)[44], but can also be attributed to immunosuppressive treatment by itself.

It was clearly shown that PCMV/PRV replicates in the transplanted heart (Fig. 1, Table 1). Already at day three an increase of the copy number in the heart was observed, reaching much higher copy numbers later. It may be suggested that the pharmaceutical immunosuppression given to prevent rejection and possibly the PCMV/PRV-induced immunosuppression plays an important role in allowing increased PCMV/PRV replication. For comparison, previous studies of the replication rate of HCMV in humans showed that its dynamics are rapid, with a doubling time of viraemia of approximately 1 day[63]. In analogy to the HCMV infection, where a clear threshold relationship was observed, e.g., the virus causes disease once a threshold value of viral load is exceeded[11], such a threshold seems also important in the case of PCMV. In this context, a viral load of 2 to 3 million copies may be tolerable until day 30–40, whereas higher viral loads further reduce the survival time.

Since other viruses could also interfere with the survival time of the transplants, activation of BaCMV in the transplanted baboon and transmission of HEV, PERV, PCV1, PCV2, as well as PLHV1, 2 and 3 was analysed. All baboons used in these investigations carried BaCMV, which is common in baboons. In one case a strong

activation of BaCMV after transplantation of a PCMV-positive pig heart was observed (baboon I)[27]. In all other cases a direct activation of BaCMV could not be observed. Concerning BaCMV activation, in another preclinical trial transplanting pig kidneys into baboons, it was also observed in control animals without transplantation, but with the corresponding immunosuppression, indicating that immunosuppression alone is able to activate BaCMV[64].

HEV, genotype 3, is a well-known zoonotic virus, it is frequently transmitted to humans by undercooked meat or contact, but also by manure-contaminated fruits and water, and it induces chronic infection in immunosuppressed patients and severe liver disease in patients with an underlying liver failure[65]. However, until now no HEV transmission was reported in all preclinical and clinical xenotransplantation trials, including this study (Table 2).

PERV DNA was observed in the circulation of baboons following transplantation of a pig heart (Table 2). However, this observation appears to be due to circulating DNA from dead transplant cells or persistent pig cell microchimerism.

PCV1 and PCV2 were not present in the donor pigs and, logically, could not be transmitted to the recipients. Meanwhile it was published, that four donor pigs (animals 5803, 5807, 6249, 6253) were infected with PCV3 and that this virus was transmitted in all four cases to the baboon recipients (baboons O, N, P, Q)[66]. PCV3 is a newly described member of the virus family *Circoviridae*, it is highly distributed among farms pigs and wild boars worldwide (for review see Reference 67). Similarly to the situation with PCV2, PCV3 was found in healthy animals as well as in animals suffering from different diseases, suggesting that coinfections with other viruses are necessary for the pathogenic potential. PCV3 does not seem to influence the survival time because baboons with the highest survival time (animal N—182 days, O—195 days) and animals with low survival times (P—15 days, Q—27 days) were infected with PCV3. In contrast, there was a clear correlation between the infection with PCMV and survival (15 and 27 days versus 182 and 195 days).

Although all donor pigs were infected with PLHV-1 and PLHV-2, no transmission to the baboon recipients was observed, PLHV-3 was not found in the donor pigs. PLHV-1, -2, and -3 belong to the subfamily Gammaherpesvirinae in the Herpesviridae family. The pathogenicity of PLHV in pigs under natural conditions is still unclear. Under experimental conditions PLHV-1 is associated with post-transplant lymphoproliferative disease (PTLD) in miniature pigs following allogeneic haematopoietic stem cell transplantation[68,69]. The clinical symptoms of experimental porcine PTLD, such as fever, lethargy, anorexia, high white blood cell count and palpable lymph nodes, are similar to those of human PTLD, a serious complication of solid organ and allogeneic bone marrow transplantation, which was linked to a human gammaherpesvirus, Epstein-Barr virus (human herpes virus-4, HHV-4)[70].

In order to prevent transmission of porcine viruses after xenotransplantation, elimination programs had been proposed which are based on selection and isolation of virus-negative animals, vaccination or treatment with an effective antiviral drug (both are not available in the case of PCMV/PRV), early weaning, colostrum deprivation, Caesarean delivery or embryo transfer. Although PCMV/PRV can be transmitted via placenta[71,72], successful elimination can be achieved by early weaning[35,73], providing virus-free animals for a safe xenotransplantation.

Methods

Animals and transplantations. All details of the donor pigs, the baboon recipients and the transplantation procedures were described in reference 1.

Immunosuppression. All baboon recipients received an immunosuppression including an induction therapy with an anti-CD20 antibody, anti-thymocyte-globulin and a monkey-specific anti-CD40 monoclonal antibody or humanized anti-CD40LPASylated as described in detail[1]. The group III baboon recipients analysed here were weaned from cortisone at an early stage and received antihypertensive treatment. In addition, a temsirolimus medication was applied.

Plasma and blood collection. Blood sampling from adult sows was performed without sedation under manual fixation. Whole blood was drawn from the jugular vein with single-use needles (Ehrhardt Medizinprodukte, Geislingen, Germany) into lithium heparin and serum Monovettes (Sarstedt, Nümbrecht, Germany). Blood from the baboons was taken using a central venous catheter.

Ethics statement. Both the generation of transgenic animals, as well as interventions on re-cloned animals, were performed with permission of the local regulatory authority. Applications were reviewed by the ethics committee according to §15 TSchG German Animal Welfare Act. The xenotransplantation experiment was approved by the Government of Upper Bavaria, Munich, Germany. Housing, feeding, environmental enrichment, and steps taken to minimise suffering, including the use of anaesthesia and method of sacrifice, was in accordance with the recommendations of the Weatherall report "The use of non-human primates in research".

Testing for PCMV/PRV. PCMV/PRV testing was performed as described[25] using specific primers (Table 3). Briefly, DNA was extracted from sera, blood and organs of the pigs using the DNeasy Blood &Tissue kit (Qiagen GmbH, Hilden, Germany). DNA was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Worcester, MA, USA). To screen for PCMV, a real-time PCR using described primers[26] and the SensiFast probe no ROX kit was performed according to supplier recommendations (Bioline GmbH, Germany). DNA from formalin-fixed tissues was extracted by using the QIAamp DNA FFPE Tissue Kit (QIAGEN), following the manufacturer's instructions. As the starting material was not paraffin-embedded but only formalin-fixed tissue, we started the process by cutting the tissue into small pieces, added buffer and proteinase and followed the manufacturer's protocol by incubating at 56 °C. A detection limit of 20 copies was determined for the reported

Primers Used for PCR	Sequence 5′–3′	Nucleotide position	Accession number	References
Primers used for PCR				
PLHV-1,-2 (747) fw PLHV-1,-2 (747) rev	CAYGGTAGTATTTATTCAGACA GATATCCTGGTACATTGGAAAG	21,146–21,167 21,488–21,467	AY170317.1	Ehlers[81]
PLHV-3 (905) fw PLHV-3 (905) rev	ACAAGAGCCTTAGGGTTCCAAACT GTGTCCAGTGTTGTAATGGATGCC	13,472–13,495 13,727–13,704	AY170316.1	Chmielewicz et al.[82]
PCV1 fw (F41) PCV1 rev (B42)	ATACGGTAGTATTGGAAAGGTAGGG ACACTCGATAAGTATGTGGCCTTCT			Mankertz et al.[84]
PCV2 fw (F66) PCV2 rev (B67)	GGTTTGTAGCCTCAGCCAAAGC GCACCTTCGGATATACTGTCAAGG	567–546 152–175	KT868491.1	Mankertz et al.[66]
PERV env C fw PERV env C rev	CTGACCTGGATTAGAACTGG ATGTTAGAGGATGGTCCTGG	6606-6625 6867–6886	AM229312	Takeuchi et al.[74]
Primers and probes used for real-time PCR				
pGAPDH fw pGAPDH rev pGAPDH probe	ACATGGCCTCCAAGGAGTAAGA GATCGAGTTGGGGCTGTGACT HEX-CCACCAACCCCAGCAAGAGCA CGC-BHQ1	1083–1104 1188–1168 1114–1137	NM_001206359.1	Duvigneau et al.[85]
JVHEVF JVHEVRJVHEVProbe	GGTGGTTTCTGGGGTGAC AGGGGTTGGTTGGATGAA FAM-TGATTCTCAGCCCTTCGC-BHQ	5261–5278 5330–5313 5284–5301	M73218	Jothikumar et al.[60]
PCMV fw PCMV rev PCMV probe	ACTTCGTCGCAGCTCATCTGA GTTCTGGGATTCCGAGGTTG 6FAM-CAGGGCGGCGGTCGAGCT C-BHQ!	45206–45226 45268–45249 45247–45229		Mueller et al.[33]
BaCMV real fw BaCMV real rev BaCMV probe	GTTTAGGGAACCGCCATTCTG GTATCCGCGTTCCAATGCA 6FAM-TCCAGCCTCCATAGCCGG GAAGG-BBQ			Mueller et al.[32]
huGAPDH fw huGAPDH rev huGAPDH probe	GGCGATGCTGGCGCTGAGTAC TGGTCCACACCCATGACGA HEX-TTCACCACCATGGAGAAGGCT GGG-BHQ1	3568–3587 3803–3783 3655–3678	AF261085	Behrendt et al.[86]
PERV Pol fw PERV Pol rev PERV Pol probe	CGACTGCCCCAAGGGTTCAA TCTCTCCTGCAAATCTGGGCC 6FAM-CACGTACTGGAGGAGGGTCAC CTG-BHQ1		HM159246	Yang et al.[8]

Table 3. Primers and probes.

PCR method[26]. Various amounts (25—250 ng) of DNA were used for testing. The reaction mixture contained 300 nM of both primers, and 250 nM of the probe (Table 3) in a final volume of 20 µL. The following conditions for amplification were used: denaturation at 95 C for 5 min, and 45 cycles of amplification with denaturation at 95 C for 15 s, annealing at 56 C for 30 s and extension at 72 °C for 30 s. Reporter fluorescence was measured using the CFX96 Toich Real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

Testing for BaCMV. To test for BaCMV, a real-time PCR was performed using described primers[32] (Table 3), but the enzymes and conditions had been changed: Denaturation at 95 °C for 5 min, and 45 cycles of amplification with denaturation at 95 °C for 15 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s.

Testing for PERV. To test for PERV, a PCR was performed using primers for the pol sequence (Table 3) as described[4]. This PCR detects PERV-A, PERV-B and PERV-C, since the pol region is highly conserved. To test for PERV-C, a PCR was performed using primers specific for the env region of PERV-C (Table 3)[74,75]. A Western blot analysis was performed using recombinant p27Gag protein, p15E protein and gp70 protein as well as goat sera against these recombinant proteins as positive control as described[4,76].

Testing for HEV. To test for HEV, a real-time PCR was performed as described[77] using specific primers[78]. Western blot analysis was performed using two overlapping recombinant ORF2 proteins of HEV genotype 3 as described[77]. On protein contained the immunodominant epitope (GT3-Ctr, aa 326–608, 32 kb)[79], the other was with a glutathione-S-transferase tag (aa452-617, 44.5 kb, Orosperc, Ness Ziona, Israel).

Testing for PLHV. To test for PLHV-1, PLHV-2 and PLHV-3 two PCR methods and specific primers (Table 3) were used as described[80–82].

Testing for PCV. To test for PCV1 and PCV2, a PCR method and specific primers (Table 3) were used as described[63], using specific primers[83].

Measurement of cytokines. IL-6 release over time was studied in the Laboratoriumsmedizin of the Munich University using an ELISA (Roche, Elecsys IL-6).

The BD™ CBA Non-Human Primate (NHP) Th1/Th2 Cytokine Kit (BD Biosciences, Heidelberg) was used to measure IL-2, IL-4, IL-5, IL-6, TNF and IFN γ in baboon serum samples according to the manufacturer´s instructions. Serum samples were diluted 1:20 before use. Samples were acquired by use of a LSR II cytometer (BD Biosciences). To measure IL-10 serum levels the IL-10 Monkey ELISA Kit (Thermo Fisher, Waltham) was used according to the manufacturer´s instructions.

Measurement of coagulation. The PAI-1/tPA complexes were measured in baboon EDTA plasma samples by a commercial ELISA kit for the measurement of human PAI-1/tPA complexes (Abcam, ab192151, https://www.abcam.com/human-pai1tpa-elisa-kit-ab192151.html).

Tissue processing and histological staining. All myocardial specimens were fixed in 4% neutral-buffered formalin and embedded in paraffin. Tissue sections were then stained with hematoxylin and eosin.

Western blot analysis. To detect antibodies against PERV in baboons, recombinant surface envelope protein gp70, transmembrane envelope protein p15E and core protein p27 were used as described[4,76]. As control goat antisera against these recombinant proteins were used. Secondary anti-goat IgG and anti-human antibodies were used and the assay was developed using alkaline phosphatase. To detect antibodies against HEV in pigs and baboons, a recombinant genotype 3 (GT3) ORF 2 core antigen[61] and a recombinant 44.5 kDa protein with a glutathione-S transferase (GST) tag fused to the ORF2 fragment (Prospec, Ness Ziona, Israel) was used[77]. Alkaline phosphatase conjugated antibodies were used and the reaction was developed using NBT (nitro-blue tetrazolium chloride)-BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) substrate.

Data availability

The data that support the findings reported herein are available on reasonable request from the corresponding author.

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Author contributions

L.K. and U.F. performed the detection methods for all viruses, M.L., B.Re., M.M., J.R., T.M., P.B. and J-M.A. performed the transplantations, collected the samples for virus testing and arranged IL-6 testing. E.W. provided the multitransgenic pigs used for xenotransplantation. B.Ro. and C.S.-H. performed the cytokine analyses, A.M., F.L., and R.R. performed the coagulation studies. C.W. performed the histological investigation, J.D. planned the virological part of the study and was writing the original draft preparation, L.K., U.F., M.L., B.Ro., J-M.A, A.M., F.L., N.S., R.R, C.W., and E.W. were reviewing and editing the manuscript, J.D., B.Re., R.R. and E.W. were supervising the project; J.D., R.R., P.B., B.Re. and E.W. were responsible for funding acquisition.

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Competing interests

The authors declare no competing interests.

Additional information

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Pig-to-non-human primate heart transplantation: The final step toward clinical xenotransplantation?

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BACKGROUND: The demand for donated human hearts far exceeds the number available. Xenotransplantation of genetically modified porcine organs provides an alternative. In 2000, an Advisory Board of the International Society for Heart and Lung Transplantation set the benchmark for commencing clinical cardiac xenotransplantation as consistent 60% survival of non-human primates after life-supporting porcine heart transplantations. Recently, we reported the stepwise optimization of pig-to-baboon orthotopic cardiac

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costimulation blockade; heart preservation; graft growth

xenotransplantation finally resulting in consistent success, with 4 recipients surviving 90 ($n = 2$), 182, and 195 days. Here, we report on 4 additional recipients, supporting the efficacy of our procedure.

RESULTS: The first 2 additional recipients succumbed to porcine cytomegalovirus (PCMV) infections on Days 15 and 27, respectively. In 2 further experiments, PCMV infections were successfully avoided, and 3-months survival was achieved. Throughout all the long-term experiments, heart, liver, and renal functions remained within normal ranges. Post-mortem cardiac diameters were slightly increased when compared with that at the time of transplantation but with no detrimental effect. There were no signs of thrombotic microangiopathy.

The current regimen enabled the prolonged survival and function of orthotopic cardiac xenografts in altogether 6 of 8 baboons, of which 4 were now added. These results exceed the threshold set by the Advisory Board of the International Society for Heart and Lung Transplantation.

CONCLUSIONS: The results of our current and previous experimental cardiac xenotransplantations together fulfill for the first time the pre-clinical efficacy suggestions. PCMV-positive donor animals must be avoided.

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Alternative sources are urgently needed to fulfill the severe shortfall in human-donated organs, such as hearts. Xenotransplantation promises a potentially unlimited supply. However, the necessary techniques are complicated and potentially risky. Thus, it has been decided that only experienced groups should attempt the first clinical trials, and these should be strictly controlled by the national and international regulatory authorities. Key issues include the means of ensuring microbiologic safety and evaluating the efficacy of the transplants in a relevant pre-clinical model.[1]

The report of the Xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation (ISHLT) was one of the first publications to deal with these topics.[2] Regarding efficacy, in December 2000, an international board comprising experts from North America, Europe, Australia, Japan, and South Africa recommended that the required standard should be approximately 60% survival in good health for at least 3 months in a life-supporting pig−to−non-human primate system. Here, almost 20 years after these advisory guidelines were made, we report on the completion of a series of experiments that has achieved this benchmark. In addition, we emphasize the importance of porcine cytomegalovirus (PCMV)−negative donor animals.

Methods

The stepwise development of our successful protocol is described in detail in our previous publication.[3] Here, we focus on the completion of our last group treated exclusively with a chimeric anti-CD40 antibody (Ab), to which we added 4 additional experiments, ending with a total of 8. These additional experiments commenced in October 2018 and lasted until May 2019. In brief, orthotopic heart transplantations were performed according to the technique of Lower and Shumway.[4] Triple genetically modified porcine donor organs (German Landrace and Large White, Blood Group 0) were generated by breeding. The donors were α1,3 galactosyl transferase deficient and hemizygous transgenic for human CD46 (hCD46) and human thrombomodulin (hTBM) (Revivicor, Blacksburg, VA, and Institute for Molecular Animal Breeding and Biotechnology, Ludwig Maximilian University, Munich, Germany). A total of 8 male captive-bred baboons (7 *Papio anubis* [Blood Groups B and AB] and 1 *Papio hamadryas* [Blood Group A], German Primate Center, Göttingen, Germany) served as recipients.

The explanted porcine hearts were perfused in a small heart-lung machine with 8°C oxygenated solution containing albumin, erythrocytes, catecholamines, and hormones.[5] Cardioprotection was also provided intermittently during the implantation procedure until the final (aortic) anastomosis commenced.

Immunosuppression followed M.M. Mohiuddin's suggestions with some modifications.[6] Induction therapy included anti-CD20 Ab, anti-thymocyte globulin, and anti-CD40 Ab. Maintenance therapy comprised mycophenolate mofetil, anti-CD40 Ab, and methylprednisolone, the latter was tapered down over 19 days to slow graft overgrowth. Temsirolimus was added for the same reason. Anti-hypertensive treatment was deemed necessary because baboons have a higher blood pressure than pigs. Additive therapy included anti-inflammatory agents such as IL-1 and IL-6 receptor antagonist, tumor necrosis factor-α inhibitor, aspirin, heparin, and ganciclovir.

A wireless telemetric transmitter (Data Science International, Boston, MA) was implanted to provide continuous aortic and left ventricular pressure measurements. Cardiac output and stroke volumes were assessed by transpulmonary thermodilution. Left and right ventricular sizes and functions were evaluated by transthoracic short-axis view echocardiography once a week. Regular hematologic data from the peripheral blood were collected. Serum measurements were used to monitor heart, kidney, and liver functions. Plasma levels of non-galactose-α1,3-galactose IgM and IgG Abs were quantified by flow cytometry.[7] During necropsy, tissue samples were collected for hematoxylin and eosin staining.

Testing of the donor pigs and the recipient baboons for PCMV was performed using real-time polymerase chain reaction as described elsewhere.[8]

The study was approved by the local authorities and the Government of Upper Bavaria. All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health and German Legislation).

Results

For this study, we completed our series of consecutive transplant experiments with 4 additional animals (altogether now 8, Figure 1).

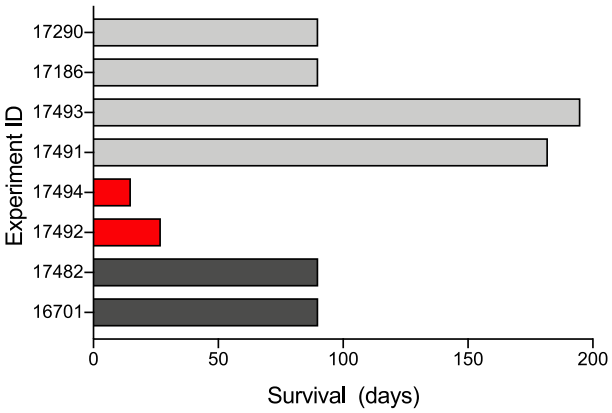

Figure 1 Survival of 8 consecutive orthotopic porcine transplants into non-human primates. The previous experiments lasting for 90 days or longer are shown in gray,[3] the 2 additional animals with PCMV-positive donor organs in red, and the 2 final experiments completing the series in black. All the animals living for 90 days or longer were euthanized in good clinical condition. PCMV, porcine cytomegalovirus.

The first 2 additional baboons that were lost prematurely had received organs of PCMV-positive donors from the same litter. These recipients succumbed to multiorgan failure on Days 15 and 27 as indicated by laboratory analyses (Table 1). For the 2 subsequent experiments, hearts from PCMV-negative donors were used. These donor animals were weaned from their mothers early after birth to prevent PCMV infection.[9] These 2 experiments lasted for the whole pre-defined period of 90 days, and the recipients were euthanized in good physical condition. Altogether, of the 8 consecutive transplants, 4 experiments were continued for 90 days and 2 for 182 and 195 days. All experiments were terminated owing to regulatory requirements.

In accordance with the previously reported outcomes, none of these 4 additional animals showed signs of hyperacute or delayed humoral rejection, including those 2 animals that had received PCMV-positive xenografts. There was no increase of anti-non-galactose-α1,3-galactose IgM or IgG (Figure 2a and b) nor any definitive increase of troponin T (Figure 3, Table 1). Platelet counts and lactate dehydrogenase values were within normal ranges, suggesting no thrombotic microangiopathy (Figure 4). Liver function tests (bilirubin, aspartate aminotransferase, prothrombin ratio, cholinesterase; Figure 5, Table 1) were normal in the long-term follow-up, as were the renal values (creatinine; Figure 5).

Comparison of pre-cardiopulmonary bypass measurements of the baboon hearts with the post-cardiopulmonary bypass measurements of the porcine-grafted hearts showed no significant differences in stroke volumes and cardiac indices (data not shown). Post-operative requirements for adrenaline and noradrenaline were low.

Weekly short-axis echocardiographic measurements revealed near-normal left and right ventricular cavities and wall thicknesses; the results of our additional experiments were in accordance with the data already published.[3]

Table 1 Serum Levels of Heart and Liver Enzymes, Platelet Counts, and Prothrombin Ratio at the End of the Experiments

Experiment	ID 17290	ID 17186	ID 17493	ID 17491	ID 17494	ID 17492	ID 17482	ID 16701	Reference
Bilirubin (mg/dl)	0.2	0.2	0.2	0.2	0.4	0.9	0.1	0.1	≤1.2
AST (U/l)	27	23	63	28	6,006	2,302	26	37	≤49
PR (%)	96	117	26	99	46	83	113	108	70–130
CHE (kU/l)	9.4	14.4	7.3	7.2	4.5	3.8	9.2	13.2	4.6–11.5
Trop T (hs) (ng/ml)	0.037	0.018	0.556	0.140	0.619	0.864	0.140	0.115	≤0.014
CK total (U/l)	143	66	461	96	1,444	40,000	155	73	≤189
LDH (U/l)	311	511	962	497	13,461	24,000	309	696	≤249
Platelets (G/L)	202	128	271	303	132	49	390	170	150–300
Survival (days)	90	90	195	182	15	27	90	90	—
Cause of euthanasia	Study end-point	Study end-point	Study end-point	Study end-point	Multiorgan failure	Multiorgan failure	Study end-point	Study end-point	—

Abbreviations: AST, aspartate aminotransferase; CHE, cholinesterase; CK, creatine kinase; LDH, lactate dehydrogenase; PR, pulmonary rehabilitation; Trop T (hs), troponin T (high sensitivity).
The complete series of 8 xenotransplantation experiments is depicted; ID 17290, ID 17186, ID 17493, and ID 17491 have been published previously[3]. All animals living for 90 days or longer were euthanized in good clinical condition.

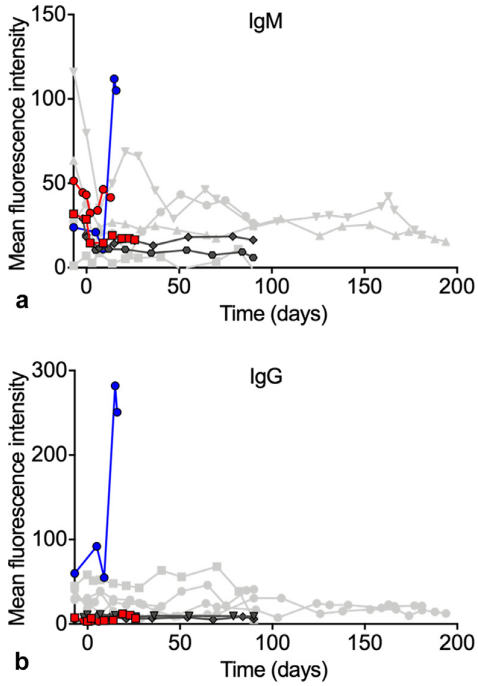

Figure 2 Levels of anti-non-galactose-α1,3-galactose xenoreactive (a) IgM and (b) IgG antibodies. The previous experiments lasting for 90 days or longer are shown in gray,[3] the 2 additional animals with PCMV-positive donor organs in red, and the 2 final experiments completing the series in black. The values from a previous baboon that rejected an intrathoracic heterotopic-transplanted pig heart served as control (blue). PCMV, porcine cytomegalovirus.

Figure 6 shows post-mortem cross sections of each of the 6 long-term porcine hearts taken at the time of euthanasia. Note the large and unrestricted diameters of the right and left ventricular cavities and their normal non-hypertrophic walls. The grafted hearts did increase in size but not enough

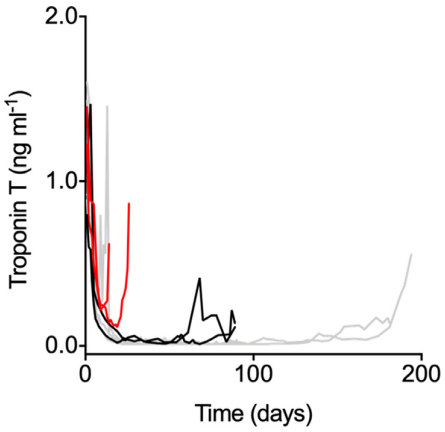

Figure 3 Serum troponin T levels increased in the immediate post-operative course but subsequently dropped to normal ranges. The previous experiments lasting for 90 days or longer are shown in gray,[3] the 2 additional animals with PCMV-positive donor organs in red, and the 2 final experiments completing the series in black. PCMV, porcine cytomegalovirus

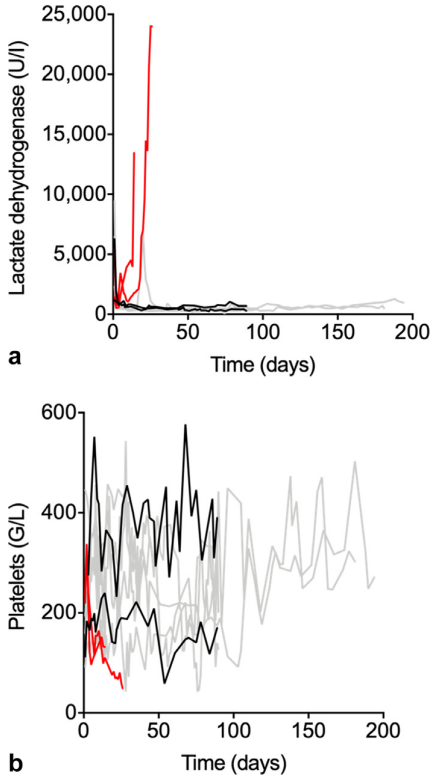

Figure 4 Exclusion of thrombotic microangiopathhy: (a) Plasma LDH and (b) platelet counts. The previous experiments lasting for 90 days or longer are shown in gray,[3] the 2 additional animals with PCMV-positive donor organs in red, and the 2 final experiments completing the series in black. LDH, lactate dehydrogenase; PCMV, porcine cytomegalovirus.

to detrimentally reduce systolic and diastolic pump functions.

Histopathologic examination of hematoxylin and eosin–stained myocardial samples revealed mild to marked perivascular and interstitial edema in all specimens (Figure 7). No larger areas of parenchymal necrosis were observed, nor were there any conspicuous vascular changes such as thrombotic lesions. On the basis of a focal inflammatory infiltrate with expansion between single myocytes and associated myocytolysis in animal ID 17186, a diagnosis was made of a limited mild acute cellular reaction involving <5% of total parenchyma examined. The infiltrate predominantly comprised activated CD3-expressing T lymphocytes admixed with a few scattered CD68-expressing monocytes and CD79a-expressing B lymphocytes and plasma cells, as shown by immunohistochemical analyses (data not shown). The other explanted hearts showed no morphologic signs of acute cellular or Ab-mediated reaction, including those that originated from PCMV-positive donors (ID 17494 and ID 17492). Together with the absence of anti-non-galactose-α1,3-galactose IgM or IgG, these results indicate that transplant rejections did not cause the demise of these 2 recipients.

Post-mortem immunohistochemical analyses of tissue samples revealed abundant expression of hCD46 and hTBM in all transplants (data not shown).

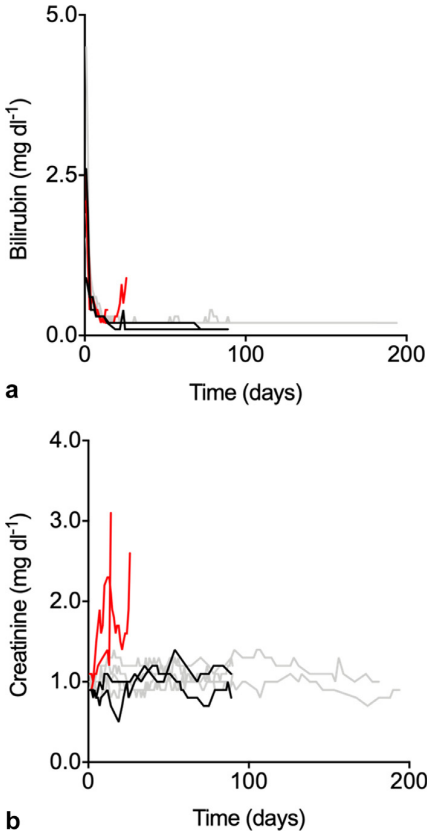

Figure 5 Liver and kidney function: (a) Bilirubin and (b) creatinine levels in serum. The previous experiments lasting for 90 days or longer are shown in gray,[3] the 2 additional animals with PCMV-positive donor organs in red, and the 2 final experiments completing the series in black. PCMV, porcine cytomegalovirus.

Discussion

We describe the first instance of 75% survival after heart replacement in baboons using triple genetically modified porcine organs, well exceeding the key requirement of the International Advisory Board of the ISHLT made in the year 2000;[2] consistent survival in 6 of 8 animals was achieved. However, we believe that more experiments will be necessary in the future—certainly more than the 10 experiments as required by the Advisory Board of the ISHLT, for example, to address the efficacy of humanized anti-CD40/CD40L costimulatory Abs[10] or similarly acting compounds[11] perhaps even for longer survival times. Irrespective of the number of pre-clinical xenotransplantation experiments performed, we have to accept that when eventually starting a clinical study, there will remain some uncertainties.[12] In the end, the advantages must far surpass the risks.

In our opinion, the following were the keys to the success of our study:

1. Stable expression of the xenoprotective transgenes in the transplants (as revealed by immunochemistry of post-mortem samples). There was no evidence of hyperacute or delayed humoral reactions nor any sign of microangiopathy in the tissue samples.
2. Non-ischemic porcine heart preservation with 8°C cold oxygenated, hyperoncotic solution containing hormones and nutrients.[5] Perfusion was continued during implantation of the grafts. Only mild (34°C) whole-body hypothermia was necessary during surgery. In contrast, static heart preservation with clinically approved crystalloid solutions such as Bretschneider's or Belzer's preparations proved detrimental, as we previously described.[3] Troponin T levels were only moderately increased after non-ischemic preservation in the immediate post-operative course; post-operatively, the levels dropped steadily to normal ranges and remained there. Transthoracic echocardiography revealed that the functions of the right and left ventricles were preserved.
3. Modified immunosuppression according to M.M. Mohiuddin,[6] with anti-CD40 Ab acting as the mainstay during induction and maintenance. There were no definite increases in anti-non-galactose-α1,3-galactose IgM or IgG. Anti-CD40 Ab is a chimeric mouse anti-rhesus/baboon protein that is possibly immunogenic and might not be safe for human use. We replaced Mohiuddin's cobra venom factor with C1-esterase inhibitor,[3] which is a clinically used and well-tolerated drug.
4. Overgrowth of grafted porcine organs due to their inherent growth potential was originally described after xenogeneic kidney transplantation[13] and was also seen in our previously reported series.[3] Overgrowth of a transplanted heart in baboon's small chest leads to endstage diastolic pump failure and subsequent lethal liver damage. Sirolimus compounds are known to control cellular growth in the body by inhibiting both mTOR kinases;[14] therefore, temsirolimus was added. Cortisone is known to cause hypertrophic cardiomyopathy early in human life,[15] so this was withdrawn as soon as possible. In addition, the mismatch between pig and baboon blood pressure was reduced by lowering the recipient baboons' higher blood pressure.[16] This combination of precautions successfully mitigated heart overgrowth, although their relative contributions to our results have not yet been rigorously assessed.
5. Avoidance of PCMV transmission would appear to be essential to prevent early recipient demise. Fortunately, this is readily achievable using early weaning of the piglets after drinking colostrum;[9] however, there are additional strategies available, such as cesarean delivery and embryo transfer,[17] to generate the animals intended for use in pre-clinical studies and clinical trials.

Thus, our data demonstrate that PCMV-negative xenogeneic orthotopic heart transplants can reliably support the life of a baboon effectively for 3 to 6 months. During that time, the porcine grafts performed normally and supported the function of other organs, as indicated by normal renal and hepatic serum parameters. Our experiments were terminated after the respective periods approved by the local authorities were reached, with the recipients still in good condition. The same combination of genetic modifications we used (α1,3-

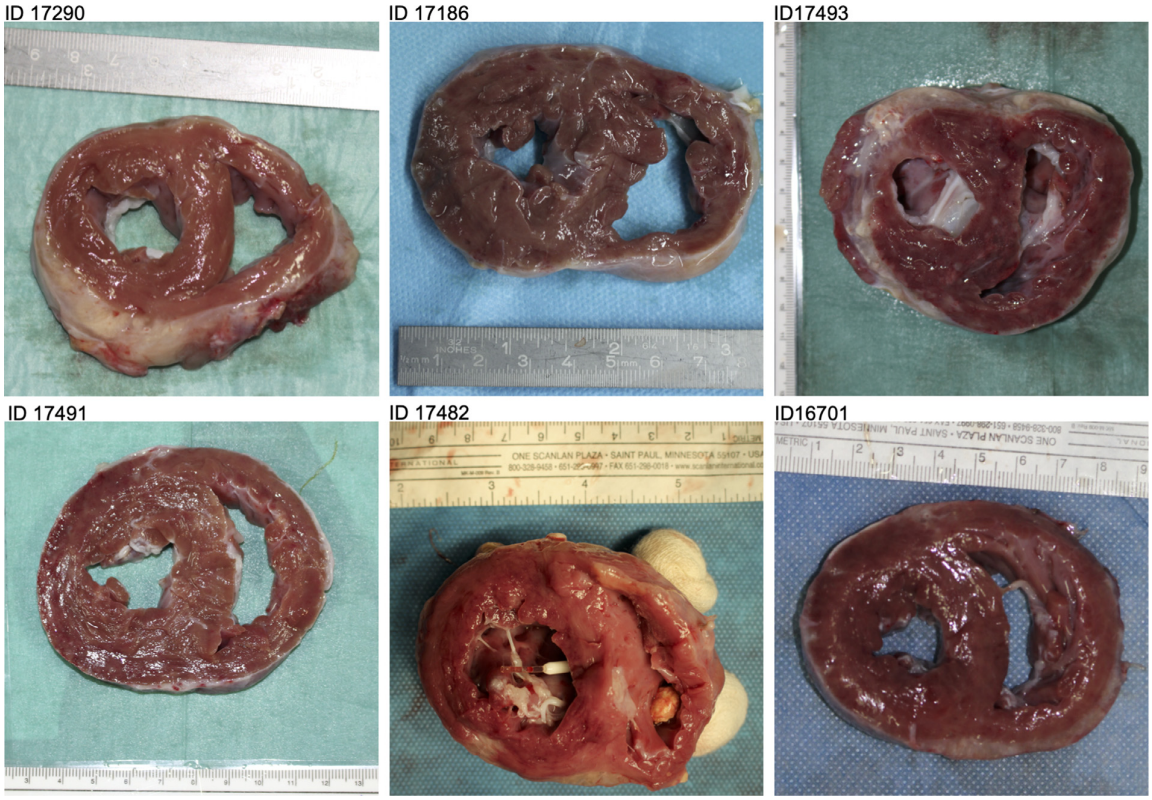

Figure 6 Cross sections of the 2 additional genetically modified porcine hearts that survived for 90 days (ID 17482 and ID 16701). For comparison, cross sections of earlier experiments from that series are shown.[3] ID 17290 and ID 17186 were euthanized in good clinical condition after 90 days. ID 17493 and ID 17491 lasted for 195 and 182 days, respectively. There are slight increases in the wall thicknesses and heart diameters, but to no hemodynamic disadvantage.

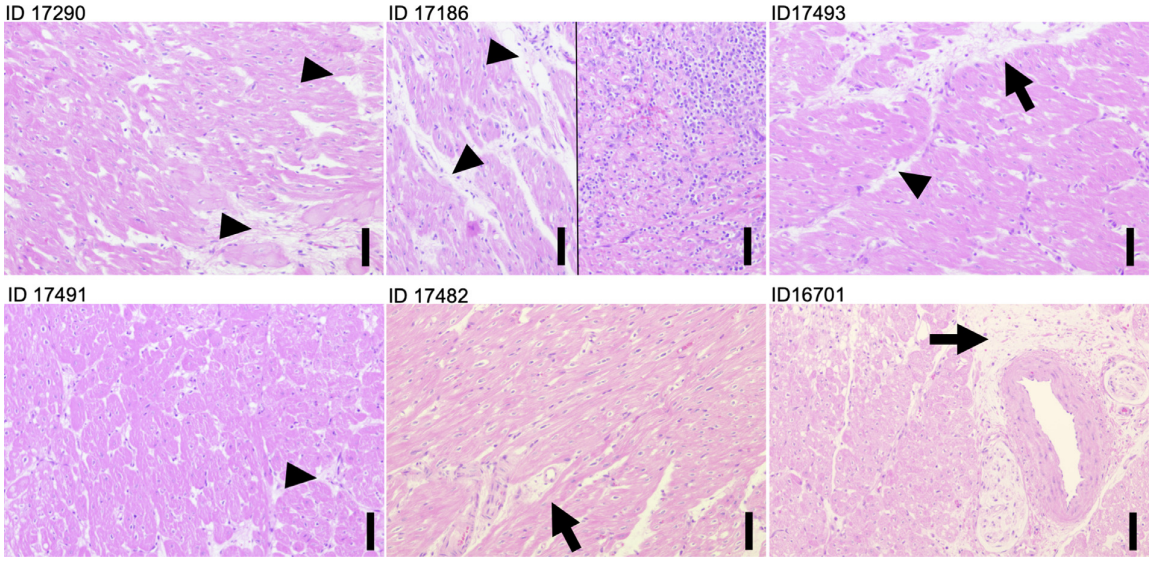

Figure 7 Microscopic evaluation revealed mild to marked perivascular (arrow) and interstitial (arrowhead) edema in specimens. On the basis of a solitary focal inflammatory infiltrate associated with myocytolysis in animal ID 17186 (right panel, upper right), a diagnosis of limited mild acute cellular reaction was made. Scale bars = 100 μm.

galactosyl transferase gene inactivation, hCD46, hTBM) has enabled porcine-grafted hearts to survive as long as 945 days (mean 298 days), although this used the less demanding, non −life-supporting heterotopic abdominal transplant technique.[6]

The mechanism of how PCMV contributes to the reduced survival of the xenotransplant is under investigation. When kidneys from PCMV-positive donors were transplanted, an upregulation of ICAM1 expression on

endothelial cells and a consumptive coagulopathy were observed.[18] The 2 additional baboons of this series that received PCMV-positive grafts had increased levels of IL-6 and tumor necrosis factor α.[19] Furthermore, high levels of tissue plasminogen activator and plasminogen activator inhibitor-1 complexes were found, suggesting a complete loss of the profibrinolytic properties of the endothelial cells.[19]

What remains to be done to gain approval for the clinical application of the much-needed xenogeneic alternative to human heart transplantation? Microbiologic safety is not an insurmountable hurdle because the microbiologic standards of European pigs under laboratory conditions are already high and elimination of PCMV from pig herds was successfully performed.[9] Methods to detect and treat a porcine endogenous retrovirus infection exist.[20] However, on the basis of the high probability of resistance development to anti-retroviral drugs, the use of animals free of porcine endogenous retrovirus C is highly recommended; porcine endogenous retrovirus A/C recombination and possible production of particles able to infect human cells[21] are, thereby, avoided. Finally, the necessary costimulation blockade with either anti-CD40 or preferably anti-CD40L Ab must be based on a humanized formulation to avoid potentially severe immunologic reactions.

Disclosure statement

D.A. is chief executive officer and chief scientific officer of Revivicor. The remaining authors have no conflicts of interest to disclose.

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