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SPOTLIGHT REVIEW

Cardiac xenotransplantation: from concept to clinic

Bruno Reichart [1], David K.C. Cooper [2,3], Matthias Längin [4], Ralf R. Tönjes [5], Richard N. Pierson III [2,3], and Eckhard Wolf [6]*

[1]Walter Brendel Centre for Experimental Medicine, Ludwig-Maximilians-Universität München, Munich 81377, Germany; [2]Center for Transplantation Sciences, Massachusetts General Hospital/Harvard Medical School, Boston, MA 02129, USA; [3]Division of Cardiac Surgery, Department of Surgery, Massachusetts General Hospital/Harvard Medical School, Boston, MA 02114, USA; [4]Department of Anaesthesiology, University Hospital, Ludwig-Maximilians-Universität München, Munich 81377, Germany; [5]Division of Medical Biotechnology, Paul-Ehrlich-Institute, Langen 63225, Germany; and [6]Gene Centre and Centre for Innovative Medical Models (CiMM), Ludwig-Maximilians-Universität München, Munich 81377, Germany

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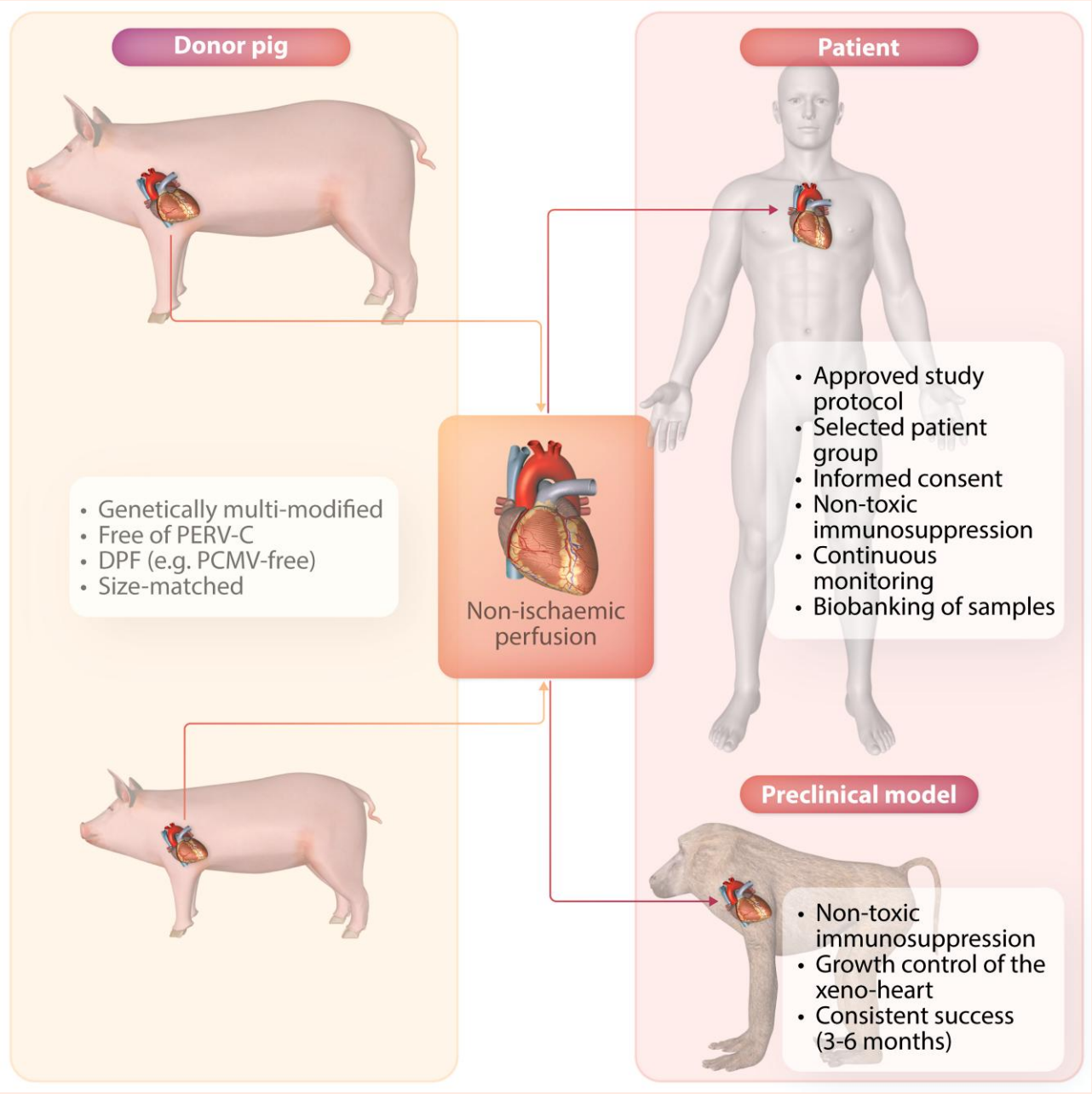
Abstract

For many patients with terminal/advanced cardiac failure, heart transplantation is the most effective, durable treatment option, and offers the best prospects for a high quality of life. The number of potentially life-saving donated human organs is far fewer than the population who could benefit from a new heart, resulting in increasing numbers of patients awaiting replacement of their failing heart, high waitlist mortality, and frequent reliance on interim mechanical support for many of those deemed among the best candidates but who are deteriorating as they wait. Currently, mechanical assist devices supporting left ventricular or biventricular heart function are the only alternative to heart transplant that is in clinical use. Unfortunately, the complication rate with mechanical assistance remains high despite advances in device design and patient selection and management, and the quality of life of the patients even with good outcomes is only moderately improved. Cardiac xenotransplantation from genetically multi-modified (GM) organ-source pigs is an emerging new option as demonstrated by the consistent long-term success of heterotopic (non-life-supporting) abdominal and life-supporting orthotopic porcine heart transplantation in baboons, and by a recent 'compassionate use' transplant of the heart from a GM pig with 10 modifications into a terminally ill patient who survived for 2 months. In this review, we discuss pig heart xenotransplantation as a concept, including pathobiological aspects related to immune rejection, coagulation dysregulation, and detrimental overgrowth of the heart, as well as GM strategies in pigs to prevent or minimize these problems. Additional topics discussed include relevant results of heterotopic and orthotopic heart transplantation experiments in the pig-to-baboon model, microbiological and virologic safety concepts, and efficacy requirements for initiating formal clinical trials. An adequate regulatory and ethical framework as well as stringent criteria for the selection of patients will be critical for the safe clinical development of cardiac xenotransplantation, which we expect will be clinically tested during the next few years.

* Corresponding author. Email: ewolf@genzentrum.lmu.de

Graphical Abstract



Keywords Heart • Non-human primate • Pig • Xenotransplantation experimental • Xenotransplantation clinical

This article is part of the Spotlight Issue on Heart Failure

1. Introduction

While state-of-the-art medical treatment for advanced heart failure is very effective,[1,2] heart transplantation (HTx) may remain the last option for patients with end-stage heart disease (reviewed previously[3–6]). However, the number of available human organs is far below the need. In 2021, 571 patients in the Eurotransplant region received a heart transplant, but 1 150 were on the active waiting list at the end of the year[7] and 126 died in 2021 while waiting for a heart.[8] In the United States, 3 817 heart transplants were performed in 2021, but 3 502 patients were still on the waiting list at year's end and 248 died waiting, whereas 946 were removed without receiving a transplant.[9]

As an option for patients who cannot receive a human heart in time, left ventricular assist devices (LVADs) or biventricular assist devices (BiVADs) have been developed. VADs have emerged as a viable 'bridge to transplantation', and as 'destination therapy' in patients who have contraindications to transplant or choose not to proceed to transplant; 78.1% of VAD recipients do not subsequently undergo heart transplants.[10] A recent

systematic review revealed excellent short-term outcomes after implantation of continuous-flow (cf) LVADs (1- and 2-year survival of 83 and 74%, respectively) but long-term survival remained limited due to the incidence of post-implantation adverse events. Particularly troublesome complications include bleeding and infection, which occur in up to 35 and 55% of patients, respectively.[11] Bleeding is related to the requirement for lifelong aggressive anti-coagulation in order to avoid thrombotic sequelae like cerebral emboli,[12,13] coupled with acquired von Willebrand factor deficiency that causes very troublesome gastrointestinal bleeding in a substantial minority of patients. Infection at the site where the electrical drive line traverses the skin of the abdominal wall can become resistant to treatment; when chronic, drive line infection greatly impairs quality of life and contributes to long-term mortality.

Right ventricular failure occurs in 40% of patients treated with an LVAD and is difficult to predict accurately. It contributes significantly to short-term mortality as well as a long-term reduction in quality and length of life among those surviving hospital discharge. Temporary additional right ventricular support systems, like Centrimag[14] or Impella,[15] are meant as a 'bridge to decision' and of course, these high-risk patients have to remain hospitalized during their treatment.

A retrospective study of 93 patients with totally implanted biventricular systems (BiVADs; which means the deployment of two separate implanted durable VADs, one for each side) reported 1-year and 2-year survival rates of 56 and 47%, results inferior to those after LVAD implantation or HTx. The most frequent adverse effect was again bleeding (35.5%), followed by infection (25.8%) and respiratory failure (20.4%).[16] In a more recent review of various BiVAD devices (including HeartWare which was withdrawn from the market[17] and the still available HeartMate 3), the median 1-year survival was 58.5%; a median rate of 31% pump thrombosis (mainly right-sided) was observed.[18]

Results after implantation of total artificial heart devices are inferior to those after implantation of BiVADs in most individual centre series and in registry reports.[19]

Cardiac tissue engineering is currently used in drug discovery science and human disease modelling (reviewed previously[20]). Moreover, cardiovascular constructs (vascular substitutes, heart valves, myocardium) with discrete structures and functions have been successfully produced, e.g. by 3D bioprinting.[21] However, the fabrication of a fully functional heart has yet to be achieved and seems perhaps decades from realization.

Therefore, xenotransplantation (XTx)—the use of animal hearts—is currently the alternative to allotransplantation that will most likely enter the clinic soon. The history of clinical cardiac XTx attempts has been reviewed recently.[22] Hearts from non-human primates (chimpanzees or baboons) survived for only few days. An exception was the transplantation of a baboon heart into an infant girl (*Baby Fae*) surviving for 20 days.[23] Although pigs are an immunologically discordant species relative to humans, they are the donor species of choice for a number of reasons[24]:

(1) Similar heart size and function as in humans.
(2) Availability of efficient and precise techniques for genetic engineering to overcome rejection mechanisms and physiological limitations.
(3) High fecundity and short time of development to sexual maturity and adult size, allowing the prospect of efficient propagation by breeding of an optimized donor pig.
(4) Natural life expectancy of 15–20 years, suggesting that clinically useful organ longevity is likely.
(5) Low risk of disease transmission when maintained under designated pathogen-free (DPF) conditions.
(6) Ethical acceptance for use as a source of potentially life-saving organs for humans.

During the last decade, remarkable progress in pig-to-primate cardiac XTx was made due to (i) an improved understanding of the underlying pathobiology; (ii) the availability of genetically tailored donor pigs with multiple genetic modifications (GMs); (iii) the introduction of perfusion preservation of the donor hearts to prevent ischemia-reperfusion injury; (iv) the optimization of pre-clinical transplantation models in baboons; (v) the development of efficacious non-nephrotoxic immunosuppressive regimens; and (vi) the development of strategies for post-implantation growth control of the xeno-heart. Based on these developments, consistent long-term function of GM pig hearts after orthotopic transplantation into baboons for up to 6 months[25,26] and recently for up to 9 months[27] has been achieved.

In January 2022, the first compassionate use of a heart from a 10-fold GM cloned donor pig for a patient with terminal heart failure was announced (highlighted previously[28]). The patient died after 2 months and it remained unclear to which extent elicited anti-pig antibodies, anti-pig antibodies contained in the intravenous immunoglobulin (IVIg) preparations administered, and graft endothelial injury associated with porcine cytomegalovirus activation in the graft may have contributed to this patient's demise ([29]; discussed previously[30]). Nevertheless, demonstration of life-supporting heart function for over 45 days is generally accepted as a proof-of-principle that clinical cardiac XTx is feasible. In this overview, we summarize the background and additional steps we feel will be required to accomplish consistent long-term success and make 'destination' xenogeneic HTx a reality.

2. Pathobiology of pig organ XTx and concepts for GM of donor pigs

The pathobiology of organ XTx is more complex than that of allotransplantation, with the innate immune response playing a greater role. The factors that contribute to xenograft destruction have been comprehensively reviewed previously[31] and so will only be summarized briefly here to provide context for the choices being discussed for pig design and recipient management.

All humans and non-human primates (NHPs) develop antibodies during infancy that cross-react with antigens present on the cell surfaces of wild-type pig cells (i.e. cells from a genetically unmodified pig). Thus, when a wild-type pig organ transplant is carried out in a human or baboon, these antibodies immediately bind to the graft vascular endothelial cells. Some bound antibodies activate the complement cascade, and others attract leucocytes which adhere and infiltrate through Fc-receptor-mediated and Fc-independent mechanisms; the graft is usually rejected within minutes to hours.[32] By general consensus, if graft failure occurs within 24 h, the phenomenon is termed 'hyperacute rejection', the histopathological features of which include venous thrombosis, loss of vascular integrity, interstitial haemorrhage, oedema, and innate immune cell infiltration (*Figure 1*).[33–35] Hyperacute or 'early' (within a few days) antibody-mediated rejection (AMR) can be delayed, but not prevented, by prior removal of anti-pig antibodies using one of several approaches: (i) by plasmapheresis, to non-specifically remove anti-pig antibodies, typically replacing lost serum proteins with plasma depleted of anti-pig antibody; (ii) by immunoadsorption against a 'sponge' organ, or a column expressing target pig donor antigens; or (iii) by infusing one or more donor antigens, to adsorb preformed anti-pig antibody. Hyperacute rejection can also be delayed or prevented by complement depletion or blockade of complement-dependent cytotoxicity, either without or, more commonly, in conjunction with addressing antibody-driven mechanisms.

Hyperacute xenograft rejection of pig organs by humans or non-human primates is mainly triggered by antibodies against galactose-α(1,3)-galactose (αGal). In addition, humans have natural antibodies against N-glycolylneuraminic acid (Neu5Gc) and a glycan corresponding to the human Sd(a) blood group antigen (often termed β4Gal). In contrast, NHPs have only anti-αGal and anti-Sd(a) antibodies (reviewed previously[36,37]). Infant primates are believed to develop antibodies to carbohydrate antigens that they do not express when their gastrointestinal tract is colonized by microorganisms expressing carbohydrate antigens which happen to be the same as those expressed on pig cells (*Figure 2A and B*).[38] To eliminate the αGal, Neu5Gc, and Sd(a) epitopes as anti-xenograft target antigens, pigs with inactivated α-1,3-galactosyltransferase (*GGTA1*), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*), and β-1,4-N-acetyl-galactosaminyl transferase 2 (*B4GALNT2*)/*B4GALNT2L* genes, so-called triple-knockout (TKO) pigs were generated as candidate pig organ donors for humans (*Table 1*, *Figure 3A*). Importantly in infant primates (including humans), the level of antibodies

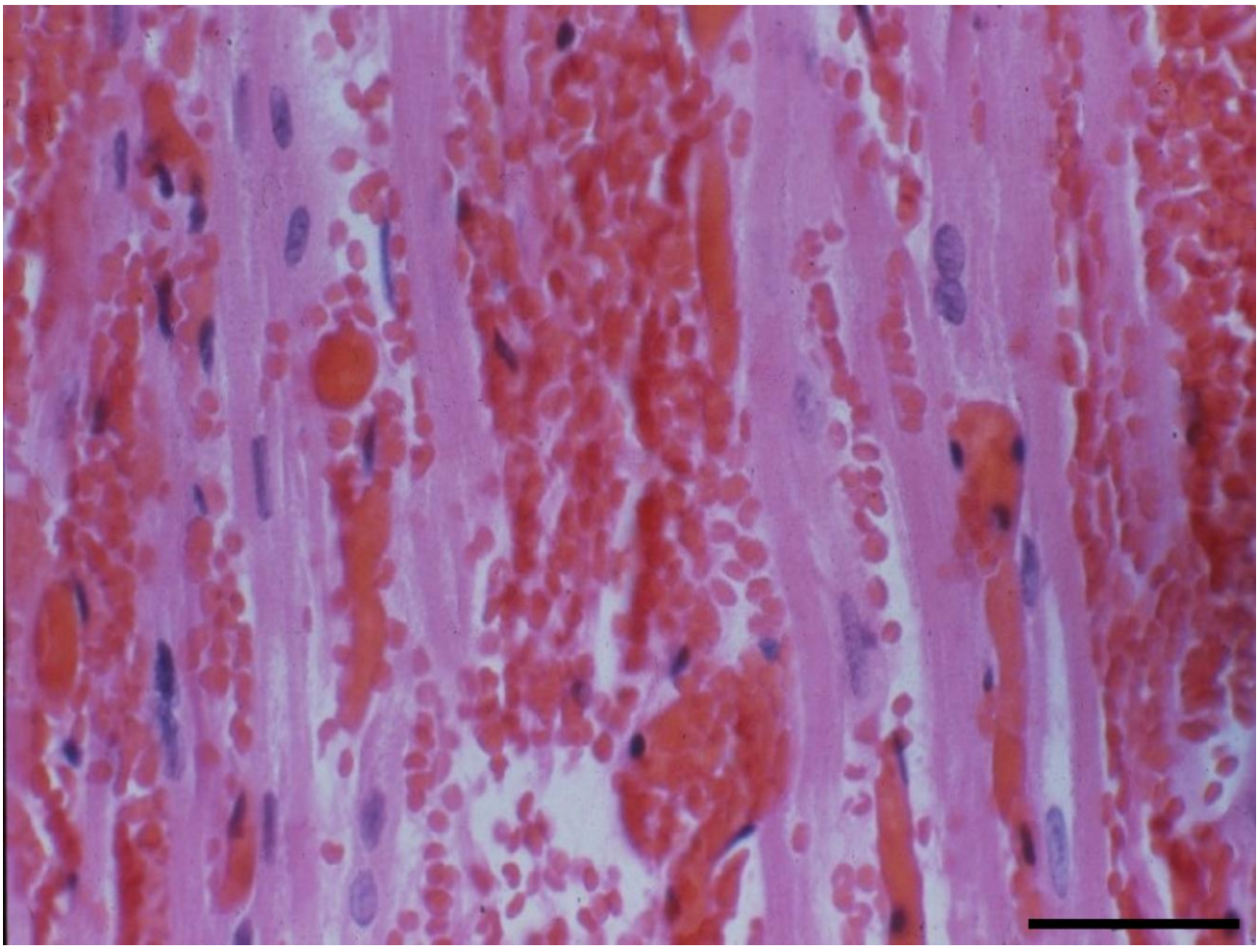


Figure 1 Histopathological features of hyperacute rejection of a wild-type pig heart after transplantation into a baboon—interstitial haemorrhage, oedema, capillary occlusion; haematoxylin & eosin (HE); bar = 50 μm.

directed against TKO pig cells is very low relative to WT or *GGTA1*-KO cells (*Figure 2C and D*). Similarly, many adult humans do not have antibodies against TKO pig cells. Based on this observation, some investigators believe that organs from TKO pigs could be sufficient to initiate clinical trials of XTx in humans with a negative CDC crossmatch against TKO pig cells.

However, complement can be activated by pathways that do not involve antibody binding, e.g. consequent to ischemia-reperfusion injury. For this reason, and to minimize the consequences of any anti-pig antibody that is either preformed in the recipient or elicited after transplantation, we believe that additional protection of the pig organ from complement-mediated injury is likely to prove beneficial to reduce xenograft injury. Protection of pig organs from complement-mediated injury has been achieved by the transgenic expression of human complement pathway regulatory proteins (CPRPs), i.e. CD46, CD55, and CD59, to inhibit the activation of the complement cascade (*Figure 3A*). Organs from pigs transgenic for one or more human CPRPs have a high degree of protection from human complement-mediated injury.[45,75] The combination of TKO and expression of human CPRPs greatly reduces pig cell injury (*Figure 4*).[76]

Elimination of the targets of anti-pig antibodies also reduces antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells. Since swine leucocyte antigen (SLA)-I cannot effectively bind inhibitory NK cell receptors, there is also a direct human NK cell cytotoxicity against porcine cells. Expression of human leucocyte antigen (HLA)-E/β2-microglobulin (B2M) in transgenic pigs is a strategy to inhibit the activation of human NK cells carrying the inhibitory receptor CD94/NKG2A[48] (*Figure 3B*). Also, macrophages are activated by porcine cells, since porcine CD47 does not bind the 'don't eat me' signal regulatory protein alpha (SIRPα) on human macrophages. Therefore, transgenic pigs expressing human CD47 have been generated (*Figure 3B*), and their cells are protected from human monocyte- or macrophage-mediated cellular cytotoxicity.[49,78]

Activation of human/NHP T cells against porcine xenotransplants occurs directly via the presentation of porcine peptides by porcine antigen-presenting cells (APCs) or indirectly via human/NHP APCs. Several costimulatory and coinhibitory signals are involved in this process (*Figure 3C*). The direct activation of T cells can be reduced by the elimination or down-regulation of SLA molecules (reviewed previously[24,79,80]). The CD40–CD40L (CD154) costimulatory signal can be blocked by treatment with antibodies (see Section 3). In addition, transgenic pigs expressing CTLA4-Ig or its higher-affinity derivative LEA29Y[50,51] have been developed to block the CD28-CD80/CD86 costimulatory pathway. A complementary approach is the expression of membrane-bound human PD-L1 on pig cells to activate the inhibitory PD1 receptor on infiltrating human or NHP leucocytes.[81] In addition, transgenic pigs harbouring a secreted monoclonal anti-human CD2 antibody construct to deplete and inhibit T cells and NK cells have been produced.[82] For LEA29Y, PD-L1, or CD2-expressing pigs, local expression is being explored as an approach to down-modulate pathogenic immunity against the organ or cellular

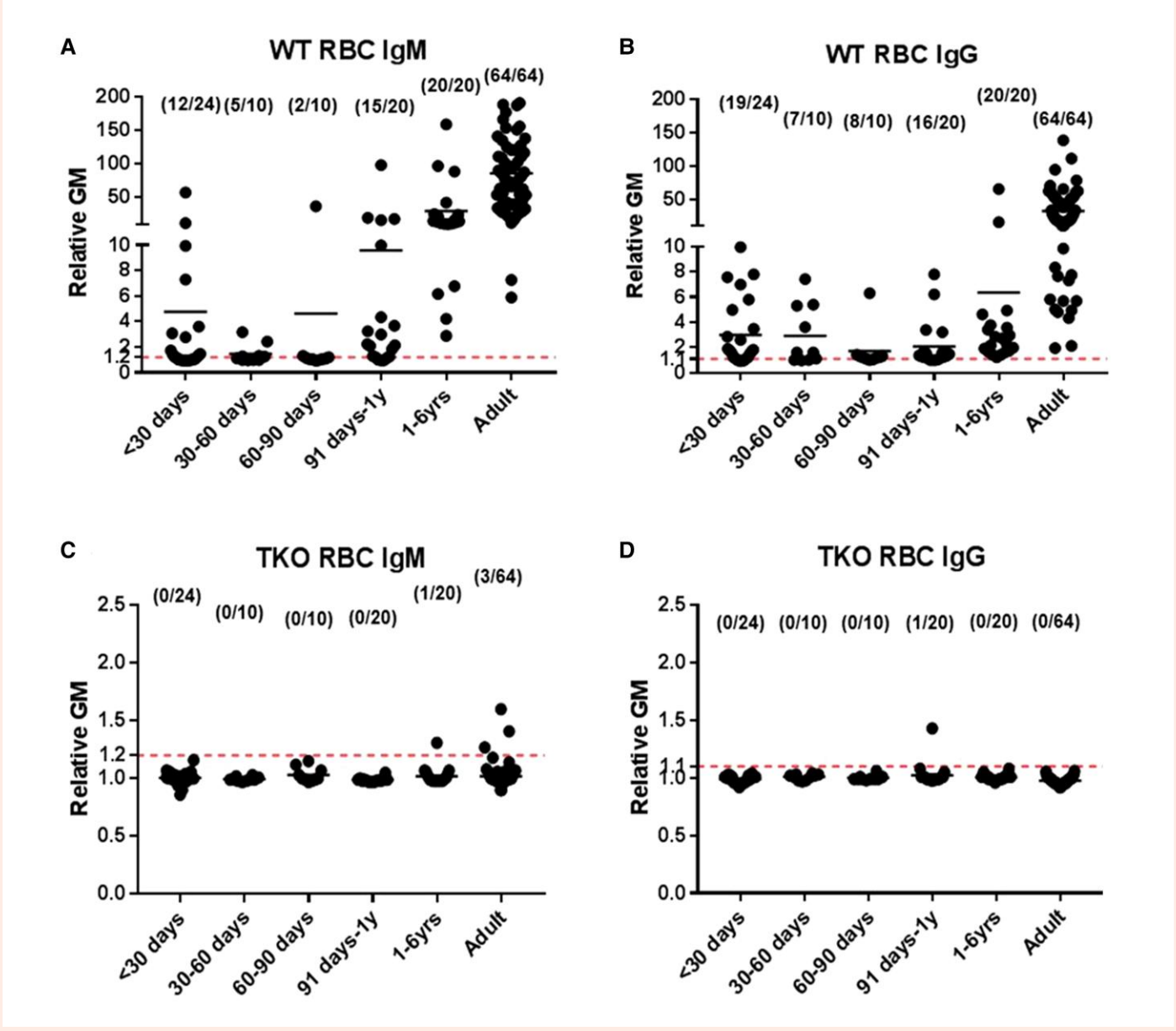


Figure 2 (*A* and *B*) Geometric mean (GM) binding and age correlation of human serum IgM (*A*) and IgG (*B*) antibodies to wild-type (WT) pig red blood cells (RBCs). There is a steady increase in IgM and IgG during the first year of life. (*C* and *D*) GM binding and age correlation of human serum IgM (*C*) and IgG (*D*) antibodies to TKO pig RBCs. There is virtually no increase in IgM or IgG antibodies during the first year of life. (Note the great difference in the scale on the *Y*-axis between top and bottom. The dotted lines indicate no IgM or IgG binding) (reproduced with permission from Li *et al.*[39]).

xenograft, and to reduce or replace the requirement for systemic immunosuppression.

Another facet of the pathobiology of pig organ XTx is coagulation pathway dysregulation (reviewed previously[83,84]). The contributing mechanisms include the immune responses described above, which trigger inflammation, vascular injury, and a procoagulant surface on the porcine endothelium, and molecular incompatibilities between porcine and human/NHP regulators of coagulation. While a systemic life-threatening consumptive coagulopathy can be avoided by the measures used to prevent hyperacute xenograft rejection, pig hearts after heterotopic abdominal transplantation in baboons showed microvascular thrombosis, or thrombotic microangiopathy (TM), even though the recipients received anti-coagulation therapy.[85,86] TM could be avoided by transgenic expression of human thrombomodulin (TBM) in the donor pigs (e.g.[87,88]), thereby overcoming the inability of porcine TBM in complex with human thrombin to promote the activation of human protein C in the anticoagulant pathway. This reaction is enhanced by the additional expression of endothelial protein C receptor (EPCR), and—while porcine EPCR appears to be functionally compatible with the human protein C pathway[89]—transgenic pigs expressing human EPCR have been produced that are expected to express higher EPCR levels and thus enhance protective thromboregulation.

Other GMs targeting coagulation dysregulation include the expression of human tissue factor (TF) pathway inhibitor (TFPI) to inhibit TF–factor VIIa complexes that initiate blood coagulation, the expression of human ectonucleoside triphosphate diphosphohydrolase 1 (CD39) that inhibits platelet aggregation and thrombus formation (reviewed previously[83]), and the siRNA-mediated knockdown of porcine TF expression.[90] In addition, transgenic pigs expressing anti-inflammatory proteins such as human

Table 1 Comprehensive list of GMs of donor pigs designed for, and potentially useful to enable, cardiac xenotransplantation

Aim/Genetic modification	Ref.
Deletion of specific carbohydrate antigens	
α-1,3-galactosyltransferase knockout (*GGTA1*-KO)	40
cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout (*CMAH*-KO)	41,42
β-1,4-N-acetyl-galactosaminyl transferase 2 knockout (*B4GALNT2*/*B4GALNT2L*-KO)	43
Expression of human complement-regulatory proteins	
human membrane cofactor protein transgenic (*hCD46*-tg)	44
human decay-accelerating factor transgenic (*hCD55*-tg)	45
human membrane inhibitor of reactive lysis transgenic (*hCD59*-tg)	46
human complement-regulatory protein C1 inhibitor transgenic (hC1-INH-tg)	47
Prevention of NK cell and macrophage activation	
HLA-E/human beta2-microglobulin transgenic (*HLA-E*/*B2M*-tg)	48
human signal regulatory protein alpha transgenic (*hCD47*-tg)	49
Prevention of T-cell activation	
human LEA29Y transgenic (*LEA29Y*-tg)	50,51
human CTLA4-Ig transgenic (*hCTLA4-Ig*-tg)	52
porcine CTLA4-Ig transgenic (*pCTLA4-Ig*-tg)	53
SLA class I KO or *B2M* KO	54–57
human dominant-negative mutant class II transactivator transgenic (*CIITA-DN*-tg) or *CIITA* mutant to reduce *SLA class II* expression	58,59
Expression of human coagulation-regulatory proteins	
human thrombomodulin transgenic (*hTBM*-tg)	60
human endothelial protein C receptor transgenic (*hEPCR*-tg)	61
human tissue factor pathway inhibitor transgenic (*hTFPI*-tg)	62
human ectonucleoside triphosphate diphosphohydrolase-1 transgenic (*hCD39*-tg)	63
human ecto-5′-nucleotidase transgenic (*hCD73*-tg)	64
Expression of anti-inflammatory proteins	
human tumour necrosis factor α–induced protein 3 (*TNFAIP3*) transgenic (*A20*-tg)	65
human haeme oxygenase 1 transgenic (*hHMOX1*-tg)	66
soluble human TNFRI-Fc transgenic (*shTNFRI-Fc*-tg)	67
Prevention of excessive growth	
Growth hormone receptor knockout (*GHR*-KO)	68–70
Reduction/elimination of the risk of PERV transmission	
Knockdown of PERV expression	71–73
Genome-wide inactivation of PERV *pol* gene	74

KO, knockout; tg, transgenic; PERV, porcine endogenous retrovirus.

TNF-alpha-induced protein 3 (TNFAIP3 alias A20)[65] or human haeme oxygenase 1 (HMOX1)[66] have been produced, hoping to prevent or diminish inflammation escaping control by other mechanism-directed GMs to the pig.

A consistent observation in pre-clinical cardiac XTx studies was a detrimental overgrowth of the xeno-heart (e.g.[25]). One idea to solve this problem was the generation of donor pigs with loss-of-function mutations of the growth hormone receptor (*GHR*) gene, which reduced their body and organ weights by about 50% without causing major metabolic disturbances ([91,92]; discussed previously[68]). A holistic proteome analysis of GHR-deficient pig hearts did not reveal signs of major molecular abnormalities.[69] Recent studies demonstrated that GHR-deficiency—among other GMs—facilitated the survival of orthotopic porcine cardiac xenografts beyond 6 months.[27,70]

A summary of GMs proposed for xeno-organ donor pigs is provided in *Table 1*. Progress in gene editing technologies facilitated the generation of pigs carrying several of these GMs (reviewed previously[93]), in some cases up to 11[94] or 12.[95] We focus here on the combinations that have been tested in heterotopic or orthotopic HTx experiments in baboons (Section 3).

3. Relevant results of heterotopic and orthotopic HTx in the pig-to-NHP model

The early results of pig HTx in NHPs (1968–2013) were comprehensively reviewed previously.[96,97] The most widely used recipient species for preclinical porcine cardiac XTx is the baboon (*Papio anubis* or *hamadryas*). In these animals, three different transplantation models have been established (reviewed previously[98]).

In the *abdominal heterotopic cardiac XTx technique,* the porcine pulmonary artery is anastomosed to the recipient inferior vena cava and the pig aorta to the recipient abdominal aorta (*Figure 5A*). After opening the vascular clamps, the transplanted heart is perfused via the coronary arteries and starts pumping, the coronary venous blood finally leaves the heart through the pulmonary artery trunk. Since there is no systemic venous return, the transplant's ventricles are not subjected to volume loading: the heart beats, but is empty except for coronary venous return, and does not support the recipient circulation. The recipient survives on his native heart, which is left untouched. This model is mainly

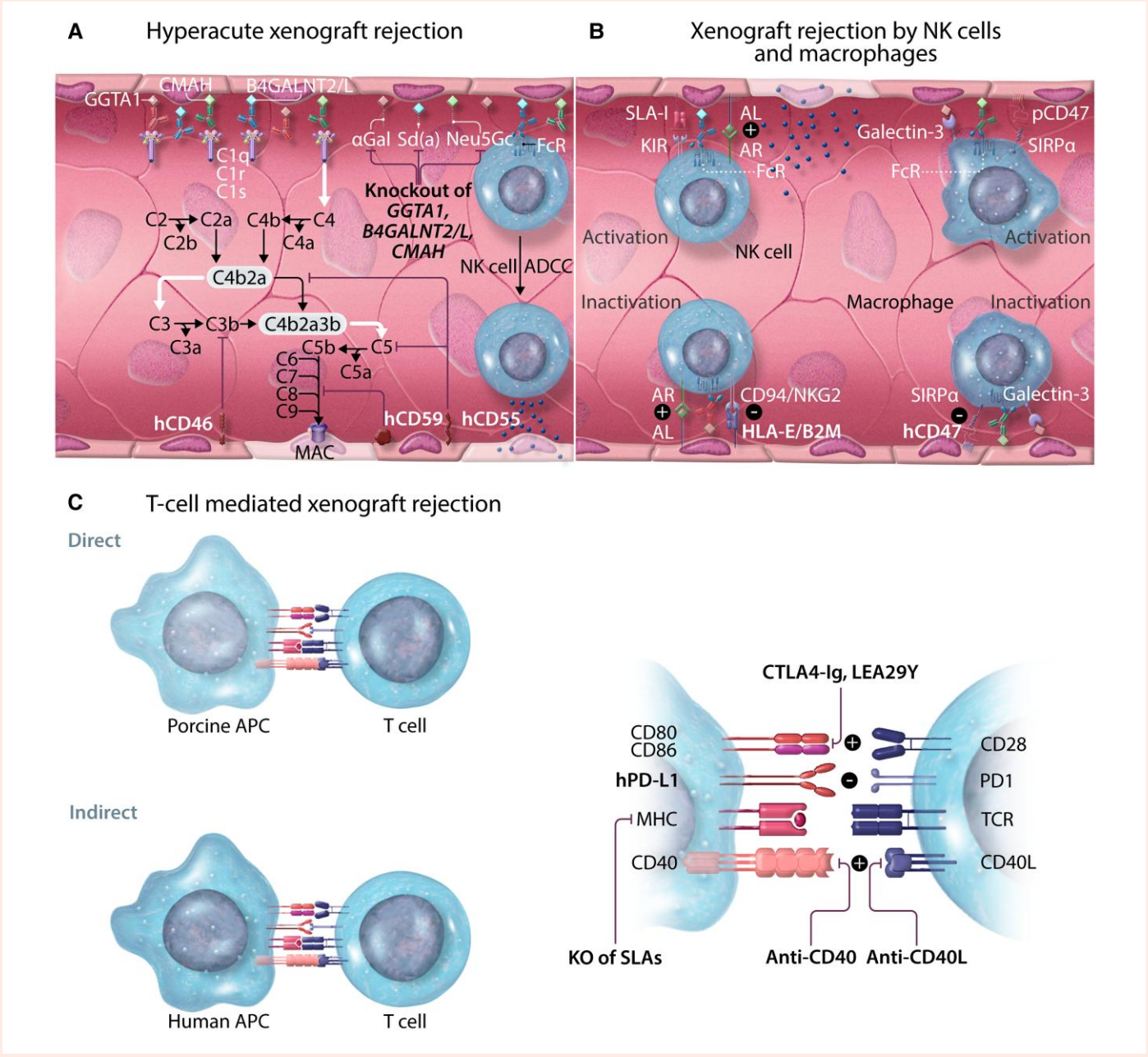


Figure 3 Mechanisms of xenograft rejection and strategies to overcome them. (*A*) Hyperacute rejection of pig-to-primate xenografts (HAR) is triggered by the binding of recipient's preformed natural antibodies to specific carbohydrate antigens [αGal, Neu5Gc, Sd(a)] on the surface of pig cells and subsequent activation of the complement system. In addition, bound antibodies activate natural killer (NK) cells via Fc-receptors (FcR) causing antibody-dependent cellular cytotoxicity (ADCC) by the release of lytic granules. In order to overcome HXR, donor pigs are genetically multi-modified to lack specific glycosyltransferases [α-1,3-galactosyltransferase (GGTA1), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), β-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2), and a recently discovered B4GALNT2-like (B4GALNT2L) enzyme] and to express one or several human (h) complement-regulatory proteins [membrane cofactor protein (CD46), decay-accelerating factor (CD55), membrane inhibitor of reactive lysis (CD59)] to prevent complement-mediated cell lysis via formation of membrane attack complexes (MACs). (*B*) Responses of NK cells and macrophages. In addition to ADCC, NK cells exhibit direct cytotoxicity of pig cells because swine leucocyte antigens (SLAs) do not effectively bind to inhibitor receptors of human/NHP NK cells (KIRs) to prevent their activation. Additionally, activating signals, resulting from activating NK cell ligands (ALs) on pig cells with their corresponding activating receptors (ARs) on primate NK cells may be involved. NK cell activation may be prevented by expressing HLA-E/beta2-microglobulin (B2M) in transgenic pigs. HLA-E binds the inhibitory NK cell receptor CD94/NKG2. Macrophages are activated by FcRs binding the Fc portion of anti-pig antibodies. In addition, they are activated by galectin-3 binding αGal on pig cells. Porcine (p) CD47 does not activate the 'don't eat me' receptor signal regulatory protein-α (SIRPα) on human macrophages. Therefore, transgenic pigs expressing hCD47 were generated to inhibit macrophage activity against xenogeneic cells. (*C*) Activation of T cells against xenotransplants may occur directly via porcine antigen-presenting cells (APCs) or indirectly via human/primate APCs presenting porcine peptides. In addition to the interaction of the peptide-presenting major histocompatibility complex (MHC) with the T-cell receptor (TCR), costimulatory signals are required, most importantly CD40—CD40L (CD154), which can be blocked by treatment with anti-CD40 and/or anti-CD40L antibodies to prevent T-cell activation. Another costimulatory pathway, CD80/CD86—CD28, can be blocked by treatment with CTLA4-Ig or its affinity-optimized variant LEA29Y. Another strategy is the involvement of the coinhibitory pathway PD1—PD-L1 expressing hPD-L1 in transgenic pigs. Finally, pigs lacking SLAs or expressing SLAs with reduced activating capacity have been produced to reduce T-cell activation via the direct pathway.

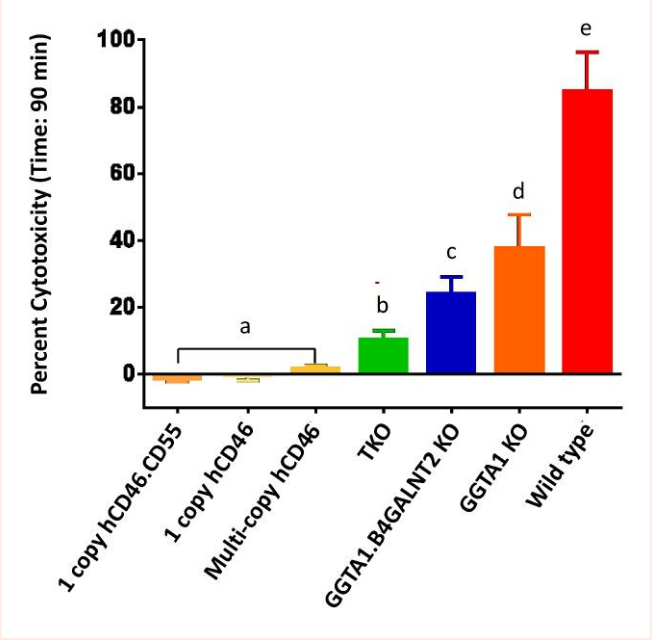


Figure 4 Effect of xenoantigen knockout and expression of complement inhibitors on serum cytotoxicity as measured by image-based complement-dependent cytotoxicity assay. Cytotoxicity decreased significantly from wild type with each additional knockout (columns with different superscripts, $P < 0.05$). Cytotoxicity was nearly eliminated when CRPs were expressed as either hCD46 alone or multi-copy hCD46, or from a single-copy bicistron composed of hCD46 and hCD55. All genotypes, except wild type, include a *GGTA1*-KO background (reproduced with permission from Eyestone *et al.*[77]).

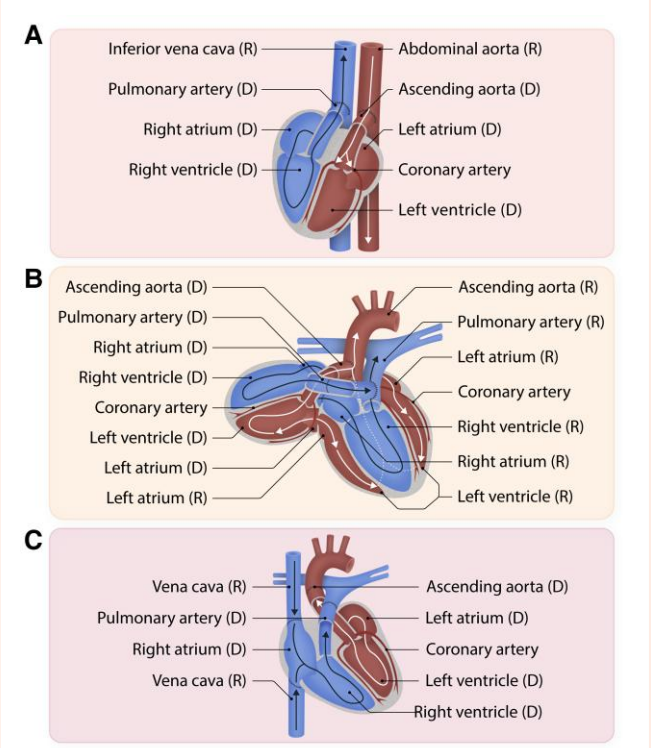


Figure 5 Models of pig-to-baboon cardiac xenotransplantation: (*A*) heterotopic abdominal, (*B*) heterotopic thoracic, (*C*) orthotopic techniques, (D) donor, and (R) recipient (modified with permission from Mohiuddin *et al.*[98]).

used to evaluate the efficacy of immunosuppressive regimens and new combinations of GMs. With appropriate immunosuppressive therapy, pig hearts lacking αGal and expressing hCD46 and hTBM have survived for up to 945 days (median 298 days) in this model ([87]; reviewed previously[99]).

In the *intrathoracic heterotopic cardiac XTx technique*, the xenograft is connected to the recipient heart in the right thoracic cavity, thus compressing parts of the upper and middle lobes of the right lung. Four anastomoses are performed to allow partial or complete life-supporting circulation: between the respective left and right atria to provide physiologically appropriate bi-atrial 'inflow'; and end-to-side 'outflow' connections of the graft to the ascending aorta and pulmonary artery trunks, the latter requiring an extension using an interposition Dacron- or Gore-Tex graft; *Figure 5B*). In this 'piggyback' position, the xeno-heart can partly or fully support the recipient's organ perfusion requirements. This technique—clinically introduced by Christiaan Barnard and his team[100–102]—has been discussed as a possible scenario for the clinical translation of cardiac XTx as the recipient's native heart can provide at least partial support as a back-up in case of xeno-heart failure.[24]

The most stringent model is the *orthotopic cardiac XTx technique*, in which the baboon heart is replaced by a pig heart using a surgical procedure identical to that of cardiac allotransplantation (*Figure 5C*).[103] This model rigorously tests the life-supporting function of the xeno-heart, and consistent success in this model is considered a prerequisite before entering clinical cardiac XTx studies.[104] Since the first orthotopic transplantation of GM pig hearts in baboons,[105] a remarkable series of additional experiments has been performed in different laboratories to optimize all remaining aspects: a consistent and well-defined phenotype of GM donor pigs; non-ischemic preservation of the heart using *ex vivo* perfusion; non-nephrotoxic immunosuppression; and post-implantation growth control of the xeno-heart (*Table 2*).

In our estimation, four major factors were essential to achieve consistent long-term survival in the orthotopic heart XTx model:

(1) GM pig hearts which are protected against hyperacute rejection and TM

While the inactivation of *GGTA1* along with expression of hCD46 and hTBM has proven sufficient to achieve intermediate-term survival in the orthotopic NHP model, the combination of inactivation of *GGTA1*, *CMAH*, and *B4GALNT2*/*B4GALNT2L* plus transgenic expression of one or several complement-regulatory proteins and human TBM is our preferred minimal set of GMs for clinical cardiac XTx studies. Testing this combination in baboons is complicated by a significant difference in the innate immune response between humans and NHPs. In contrast to humans, all Old-World monkeys, including baboons, express Neu5Gc, as do pigs. When Neu5Gc is deleted in TKO pigs, it appears that another xenoantigen (sometimes known as the '4th xenoantigen', presumed a glycan) is exposed. The structure and identity of the '4th xenoantigen' remains unknown, but most NHPs have natural antibodies against *CMAH*-KO or TKO cells.[43] Binding of these antibodies to the TKO pig graft is associated with a high level of complement-dependent cytotoxicity[111–113] and reduced graft survival in heart[27] and kidney[114] XTx models. This clinically irrelevant phenomenon has proved to be a major barrier to using NHPs to model how a TKO pig organ transplanted into a human recipient would behave.[114,115] We conclude that inactivation of the *CMAH* gene reduces the antigenicity of pig cells to human serum, as expected, and should be

Table 2 Orthotopic xenogeneic heart transplantation experiments in baboons

Genetically modified donor pigs										Organ preservation	ISR	Growth control[a]	Survival time [days][b]	Ref.
Knockouts					Transgenes									
GGTA1	CMAH	B4GALNT2(L)	GHR	hCD46	hCD55	hTBM	hEPCR	hCD47	hHMOX1					
					X					Static	A	–	<1 (2); 5 (2); 9	105
					X					Static	A	–	<1 (5); 4; 5 (3); 9	106
					X					Static	B	–	39	107
					X					Static	C	–	9; 25	108
				X						Static	C	–	0; 2 (2); 34; 40; 57	108
X					X					Static	C	–	0; 1; 2; 14	108
X				X		X				Static	D	–	1 (3); 3; 30	25
X				X		X				Perfusion	D	–	4[c]; 18; 27; 40	25
X				X		X				Perfusion	D	+	90[d] (2); 182; 195	25
X				X		X				Perfusion	D	+	15[e]; 27[e]; 90[d] (2)	26
X				X		X				Blood cardioplegia	E	–	<1; 4; 29	27
X				X		X				Perfusion	E		57	27
X				X	X					Perfusion	E	–	6	27
X	X	X		X	X					Perfusion	E	–	<1	27
X	X	X								Perfusion	E	–	8	27
X		X		X	X	X	X	X	X	Perfusion	E	–	84; 95	27
X		X	X	X		X	X	X		Perfusion	E	–	182[f]; 264	27
X					X					Static (UW)	F	+	<1	109,110
X					X					Static (del Nido)	F	+	90	109,110
X				X		X				Static (del Nido)	F	+	<1; 241	109,110

Immunosuppressive regimens (ISR): A: cyclosporine A (CsA), corticosteroids (Cs), cyclophosphamide (CyP). B: CsA, Cs, CyP, mycophenolate mofetil (MMF). C: anti-thymocyte globulin (ATG), rituximab, tacrolimus, sirolimus, TPC (αGal polymer) or GAS914 some recipients. D: induction: anti-CD20 monoclonal antibody (mAb), anti-CD40 mAb or PASylated anti-CD154 Fab, ATG; maintenance: MMF, anti-CD40 mAb or PASylated anti-CD154 Fab, methylprednisolone (MP; tapered down); anti-inflammatory therapy: interleukin-1 receptor (IL1R) blocker, IL6R blocker, tumour necrosis factor alpha (TNFA) inhibitor. E: induction: anti-CD20 mAb, anti-CD40 mAb, ATG, cobra venom factor (CVF) or C1-esterase inhibitor; maintenance: MMF, anti-CD40 mAb; anti-inflammatory therapy: IL6R blocker, TNFA inhibitor. F: induction: ATG, anti-CD20 mAb, C1-esterase inhibitor; maintenance: anti-CD40 mAb, rapamycin, methylprednisolone; adjunctive medication: etanercept (TNF-α antagonist), ganciclovir, famotidine, aspirin, bactrim, erythropoietin, triiodothyronine.

[a]Treatment with mTOR inhibitor temsirolimus or rapamycin.

[b]Numbers of animals with the same survival time are given in brackets.

[c]Technical failure.

[d]Terminated according to experimental protocol.

[e]Donor infected with porcine cytomegalovirus (PCMV).

[f]Elective euthanasia.

included in pigs intended for clinical use, but should be avoided for pre-clinical trials ([43,113]; reviewed previously[116]).

Based on the observations after orthotopic cardiac XTx in baboons, consistent expression of one complement and one coagulation pathway regulatory protein appears to be sufficient, and there is no clear evidence of a requirement to include hEPCR, hCD47, and hHMOX1, although they may turn out to be valuable.

(2) Development of a non-nephrotoxic immunosuppressive regimen involving CD40–CD154 co-stimulation blockade

Initial pig-to-baboon cardiac XTx studies used conventional immunosuppressive regimens including cyclophosphamide, cyclosporine A or tacrolimus, mycophenolate mofetil, and corticosteroids. Since 2000, co-stimulation blockade—first with anti-CD154 mAb—was used in heterotopic HTx experiments ([117]; reviewed previously[118]). Since anti-CD154 mAb was found to be thrombogenic in humans, anti-CD40 mAb-based regimens were established and have contributed to the longest reported xenograft survivals of pig hearts after heterotopic[87] and orthotopic transplantation in baboons.[25,27] The anti-CD40 antibody KPL-404 (Kiniksa Pharmaceuticals) was used in the recent compassionate transplantation of a GM pig heart in a patient.[29] It is important to note that CD40 and CD154 are differently expressed by the cell types involved in xenograft rejection: Dendritic cells, B cells, macrophages, and endothelial cells constitutively express CD40; only activated CD4+ T helper cells, CD8 + cytotoxic T cells, monocytes, and non-activated platelets express CD154 (reviewed previously[119]). New antibodies, including structurally modified, non-thrombogenic versions of anti-CD154,[120] are in clinical development (reviewed previously[121,122]).

Importantly, although blockade of the CD80/CD86-CD28 pathway using CTLA4-Ig or related molecules had some effect *in vitro*, it was insufficient *in vivo* in the pig-to-baboon model.[88,123] Since 2000, therefore, almost all successful *in vivo* studies have been based on an anti-CD40[25,27,87] or anti-CD154 agent.[120,124] Although only tested in one baboon to date, the important role of these agents (in this case anti-CD40 mAb 2C10) in maintaining a GM organ graft was demonstrated recently by prolonged graft survival from 2 to 4 months after XTx when all other immunosuppressive therapy was discontinued.[125]

(3) Perfusion preservation of the donor heart

Initially, the results of orthotopic xenogeneic HTx in baboons were inconsistent and unpredictable with 40–60% perioperative mortality, despite the use of clinically approved preservation techniques (reviewed previously[99]). This phenomenon was termed 'Perioperative Cardiac Xenograft Dysfunction' (PCXD) and was thought to be due to ischaemia reperfusion injury.[98,126] PCXD has been consistently prevented by perfusing the grafts with an 8°C hyperoncotic cardioplegic solution containing erythrocytes, nutrition, and hormones[127,128]; perfusion was intermittently continued during implantation.[25,26,128] Perfusion preservation of the donor heart to minimize graft ischaemia was also employed in the first compassionate use of a GM pig heart for a terminally ill patient.[29]

(4) Post-implantation growth control of the xeno-heart.

Domestic pig breeds used for XTx experiments, such as Landrace or Large White, attain total body weight (TBW) of 200–300 kg when fully grown. Organ sizes increase proportionally to TBW, although a recent study suggests cardiac sizes become disproportionally smaller once a TBW of 150 kg is surpassed.[129] Having reached 150 kg TBW, a porcine heart is twice as large as that of an adult human and 6 times as large as that of an adult baboon (600 vs. 300 vs. 100 g). This size mismatch is of great importance both for pre-clinical experiments as well as the clinical application of cardiac XTx.

For many years, it was believed that after xenogeneic transplantation, the graft would adapt to the growth regulation of the recipient under the influence of extrinsic (recipient-dependent) factors such as hormones and growth factors (reviewed previously[130,131]). Yet some 90 years ago, Twitty and colleagues demonstrated that the growth of organs after interspecies transplantation is (mostly) defined by intrinsic factors, i.e. genetic determination.[132] In their experiments, the transplanted organs attained sizes characteristic of the donor species. Intrinsic organ growth regulation was also observed in allogeneic and xenogeneic kidney transplantation experiments.[133,134]

After pig-to-baboon heart XTx, graft overgrowth caused a reduction in pulmonary function in the heterotopic thoracic model,[102] and diastolic pump failure and subsequent congestive liver damage in the orthotopic model.[25] In a recent study, physiological differences in afterload parameters (arterial blood pressure, systemic vascular resistance) have been described to be additionally responsible for myocardial hypertrophy and diastolic heart failure besides the obvious size mismatch of swine and NHPs.[135]

Cardiac overgrowth was successfully prevented by decreasing the blood pressure (baboons have a higher blood pressure than pigs), early weaning from cortisone, and treatment with Sirolimus or the prodrug Temsirolimus, which inhibit activation of the mechanistic target of rapamycin (mTOR) and thereby cardiomyocyte hypertrophy.[25] An alternative is the use of donor pigs with a *GHR* knockout, which reduces body and organ sizes (except brain) to roughly 50% of wild type.[69,91] Hearts from *GHR* knockout pigs did not show the characteristic hypertrophic changes after orthotopic transplantation in baboons.[27] An alternative, of course, is to use a miniature swine, e.g. Yucatan, as the basis for genetic manipulation.[120] Another option is Auckland Island pigs, which have adult organ sizes matching those of humans. Moreover, this breed includes animals free of porcine endogenous retrovirus type C (PERV-C; Olga Garkavenko and Joachim Denner, personal communication), a proposed regulatory requirement for clinical XTx studies (reviewed previously[136]).

4. Monitoring of pig heart function after orthotopic XTx

4.1 How does a healthy pig heart function compared with a healthy human/baboon heart?

The porcine heart is similar to the human heart in most anatomical aspects, but not identical.[137] There are several specific differences important for XTx surgery: in swine, a prominent left azygous vein exists and is drained via the coronary sinus; the left atrium receives 5–7 pulmonary veins[138] instead of four as observed in man; the porcine superior and inferior caval veins open into the atrium in right angles, whereas in man the orifices are in line.[139] Regarding function, the heart of a healthy swine is mostly comparable with that of a healthy human. Thein and Hammer[140] reported that cardiac output, stroke volume, heart rate, and myocardial flow are almost identical in adult pigs and humans. Also, the mean arterial blood pressure and oxygen-binding capacity of the blood are similar. The main differences between the two species are their systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR), which are twice as high in full-grown pigs as in humans. This might facilitate the porcine heart's function after transplantation into the upright human, where the heart would need to keep up blood circulation over a height of approximately 1.50 m compared with 0.50 m in the horizontally postured pig.[140] However, Thein and Hammer compared individuals of different sizes; when taking the body surface into account, the systemic vascular resistance index (SVRI) would approximately be the same in pigs and men.

For pre-clinical studies, NHPs (such as *Papio* sp.) are typically used as recipients for XTx experiments.[104] Bert *et al.*[141] found high structural and quantitative similarities between the healthy baboon and human hearts in echocardiography studies; specific exceptions are an elevated left ventricular mass in baboons, low pulmonary vascular resistance, thickened walls of pulmonary artery and aorta, an oversized mitral valve orifice and a very large left coronary artery. As baboons are much smaller than humans (20 vs. 75 kg), juvenile swine must be used as organ donors for XTx experiments. Baboons have comparable cardiac outputs, stroke

volumes, and heart rates, but 60% higher arterial pressures than size-matched piglets[135]; the SVRI of a healthy baboon is comparable with that of a man, but more than twice as high as compared with piglets. Apparently, the SVRI is lower in juvenile pigs and increases with age, similar to men.[142] Volumetric parameters of cardiac load derived by transpulmonary thermodilution, such as global end-diastolic volume, are different between the two species and do not fall into human reference values,[135] thus they need to be used with caution for perioperative goal-directed therapy.

4.2 Indicators of rejection

In the early days of XTx research, a fall in the platelet count and in serum fibrinogen in the NHP recipient indicated the development of a TM within the graft and consumptive coagulopathy in the recipient.[143] With the transgenic expression of human coagulation-regulatory proteins in the organ-source pig, this complication is now rarely seen, but these parameters should still be monitored. (On occasions, when the pig organ inadvertently does not express all of the human 'protective' proteins, which can occur after cloning, then reductions in platelet count and serum fibrinogen may well be seen.) Today, however, rejection or impending graft failure is more likely to be indicated by an increase in troponin (suggesting thrombotic complications within the graft) and/or a deterioration of graft function as seen in transthoracic echocardiography.[109]

Immune monitoring has been disappointing in predicting or even confirming rejection.[144] There may be no increase in serum anti-pig antibody levels because the antibodies are binding to the graft. However, if a significant increase in antibody levels is documented, this would support a diagnosis of AMR. The T- and B-cell counts may remain unchanged. To our knowledge, changes in serum cytokine levels have not been carefully studied.

Several new diagnostic approaches based on the detection of cell-free nucleic acids from the transplant in the recipient's circulation are currently being investigated in allotransplantation.[145–147] This approach should also work for XTx with the advantage that the origin of circulating nucleic acids from the transplant can be identified more easily due to the higher degree of sequence divergence.

4.3 Prospects for treatment of acute AMR

AMR can develop rapidly over the course of just 2 or 3 days. In our experience, attempts to reverse AMR using high-dose steroid therapy have proven uniformly unsuccessful. We suggest, therefore, that attention must be directed to safely prevent AMR (see chapter 2, *Figure 3*) as well as towards development and testing in pre-clinical models of potential AMR treatment options. These topics have been explored in allosensitized patients undergoing kidney allotransplantation,[148] and we will continue to learn from this increasing experience. Plasmapheresis and the administration of IVIg play important roles in the allosensitized patient, but plasmapheresis and immunoadsorption are difficult, though not impossible,[149] to test in small NHPs. IVIg therapy carries the risk of infusing the recipient with anti-pig antibodies unless the product is adsorbed against donor cells or through a donor-phenotype organ prior to infusion.[150] The application of these approaches will prove much easier in human patients than in experimental NHPs.

Jordan and his colleagues[148] have explored several approaches to prevent and/or treat AMR: (i) anti-plasma cell therapy (e.g. proteasome inhibitors), (ii) IL6 and IL6R inhibitors, (iii) complement inhibition, and (iv) IgG-degrading enzymes. These approaches have not yet been fully explored in XTx. It is likely that a combination of these approaches may be required to prevent and/or reverse AMR of a pig xenograft.

5. What experimental results would justify a formal clinical trial?

National regulatory bodies have the authority to determine what experimental benchmarks in pre-clinical studies are appropriate as the basis for approving clinical trials of XTx. However, with regard to cardiac XTx, we would suggest that the expectation from experimental studies in pig-to-NHP models should not be too high, based on these considerations:

(1) Some patient populations are unlikely to have timely access to a human organ donor, considering the number of potentially suitable organs that become available each year for that disease category, blood type, anti-human antibody sensitization level, and other recipient demographics.
(2) Expected outcomes (survival duration, likely complications, and expected quality of life) associated with 'destination therapy' or 'bridge to transplant' with a mechanical circulatory device are very poor for some patient populations, including infants with congenital heart disease.
(3) The '4th xenoantigen' greatly complicates interpretation of experiments using the pig-to-NHP experimental model to test organs from pigs including the TKO genotype.[111,112]

Given that numerous patients who can be expected to die on the waitlist might benefit from a successful organ xenograft and based on our current knowledge and available tools, we feel that a strong case can be made for the 'compassionate' implantation of a GM pig heart in a few well-selected candidates.

In 2000, the *ad hoc* Xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation (of which two of us were members) recommended that consistent survival of NHPs supported by pig orthotopic heart transplants for 3 months would be sufficient to warrant moving to a clinical trial.[104] That recommendation was made at a time when, because of the unavailability of pigs with adequate GMs and the inadequacy of the immunosuppressive therapy available to us, achieving even 3-month survival had been unobtainable. The state of science has changed dramatically since those pioneering days, and consequently the experimental evidence suggesting the likely success of a clinical trial needs to be stronger, and indeed is already being approached.[25–27]

We therefore suggest that (allowing for complications that are inevitably met in the pig-to-NHP model) consistent survival of up to 6 months, in the absence of features of irreversible rejection or infection, would be sufficient to warrant moving towards a clinical trial in carefully selected patients. Achieving survival for longer durations, with at least one or two recipients being followed for at least 9 or even 12 months, would be reassuring to investigators, potential recipients, and regulators, but is not a substitute for clinical experience to inform future directions. Evidence for the absence of graft injury could be confirmed by low serum troponin measurements, absence of circulating pig cell-free DNA, preserved graft morphology and function by transthoracic echocardiography, and evaluation of heart morphology and histology on post-mortem examinations. A 'clinically acceptable' immunosuppressive regimen would be expected to yield good clinical condition of the NHP recipients (i.e. normal activity, appetite, and age-appropriate weight gain as well as preserved biochemical and histologic indices of end-organ function) in the absence of serious non-cardiac complications or comorbidities.

Studies in an experimental model that (in view of the '4th xenoantigen') does not accurately reflect the clinical situation are likely to overestimate the remaining barriers to clinical success,[151,152] and managing immunosuppressed NHPs under laboratory conditions is significantly more difficult than managing a human patient in a hospital setting.

Based on our very encouraging pre-clinical results, we firmly believe that the time has come to move into the clinic. We feel we are at a stage when truly significant progress can be made in small, carefully conducted clinical trials. However, we would emphasize that any clinical trial should be carried out by a team with both experiences of clinical HTx and of orthotopic HTx in the pig-to-NHP model.

Intrathoracic heterotopic HTx (*Figure 5B*) is one possible choice in the early stages of clinical xenogeneic HTx, since the patient´s own heart may keep a recipient alive in case the xenograft exhibits transient dysfunction or fails. This technique was clinically introduced[100,153] when primary allograft failure was a common problem. Under those clinical conditions,

the transplanted left ventricle typically supported on average 73% of the total cardiac output.[154] Long-term results were good for that era, although post-operative anti-coagulation was mandatory to avoid clot formation within the recipient left ventricle and systemic thromboembolism.[155] Our group (B.R., M.L., and E.W.) carried out consecutive pig-to-baboon heterotopic heart xenograft experiments between 2009 and 2013. Short-term results (recipient survival, initial xenograft function) were excellent, but long-term results were limited due to the toxic immunosuppressive therapy under study at that time (before co-stimulation blockade was available),[102] and before the problem of intrinsic donor organ overgrowth was identified and controlled. In our view, based on the recent Maryland experience orthotopic trials are equally justifiable and technically simpler.

6. Selection of patients

It should be noted that, to date, no NHP has survived longer than 9 months after being supported by an orthotopic pig heart transplant.[25,27] Consequently, regulatory authorities like the FDA or EMA may be of the opinion that pig HTx should initially be offered as a bridge, e.g. for several months, when a cardiac allotransplantation could subsequently be performed if clinically indicated. Initial candidates could include patients who are poor candidates for mechanical circulatory support (hypertrophic cardiomyopathy, prior mechanical valve replacements, deteriorated aortic bioprothesis, post-infarct VSD). Such patients might become candidates for a xenograft 'bridge' to allotransplantation due to the high risk of death before an allograft becomes available due to increasingly unstable arrythmia burden or inotrope requirement, especially those with a high panel-reactive antibody (PRA). Some patients with a relatively recently treated malignancy might also be bridged to future consideration of an allograft. Among elderly patients with high PRA, particularly those with risk factors for poor outcomes after heart allotransplantation (reoperative status, potentially reversible renal or liver dysfunction, progressive debility likely attributable primarily to heart failure), the consent process should anticipate that the xenograft is intended as a definitive ('destination') transplantation option without necessarily excluding reconsideration of candidacy for a subsequent allograft. With the experience gained from bridging, the potential for destination therapy will become clearer. The majority of us, however, advocate for testing heart xenografts as 'destination therapy' in recipients for whom an allograft is unlikely to be feasible, including patients who are highly sensitized against human alloantigens but lack anti-pig antibodies reactive to the intended source pig.

The first patients for clinical cardiac XTx trials must be carefully selected to justify this intervention and ensure favourable outcomes. In general, intensive care unit (ICU)-dependent patients with end-stage heart failure requiring continuous intravenous catecholamines are good candidates; secondary liver and kidney damage must be considered likely reversible, and pulmonary hypertension medically treatable (reviewed previously[24]). Potential indications for the initial clinical trials of pig HTx are summarized in *Table 3*.

Table 3 Potential indications for the initial clinical trials of pig heart transplantation[a]

1. Relative or absolute contraindications to mechanical circulatory support, e.g.
   (a) restrictive or hypertrophic cardiomyopathy
   (b) presence of a dysfunctional mechanical valve prosthesis or degenerated bioprosthesis
   (c) atrial or ventricular septal defect
2. High titres of broadly panel-reactive anti-HLA antibodies (high PRA) that do not cross-react with swine leucocyte antigens (SLA)
3. Chronic rejection after cardiac allotransplantation
4. Infants and children with complex congenital heart disease, e.g. after atrial correction of a transposition of the great arteries, single-ventricle circulation after right ventricular Fontan procedures

[a]Based on Chaban *et al.*[22]

Of these, we have a special enthusiasm for using heart xenografts to address the unmet needs of paediatric patients with complex congenital heart disease, particularly those with single right ventricular physiology. Although palliative surgical techniques (Norwood, Fontan) provide adequate palliation in some patients, survival and quality of life are limited, particularly in patients with high-risk anatomic lesions or complex arrhythmias. In contradistinction, these high-risk patients do well after allotransplantation,[156] but have a high mortality while waiting for a suitably-sized heart from a deceased human donor, particularly if they are sensitized to alloantigens after implantation of a homograft for reconstruction of their original cardiac pathology.[157] In addition, mechanical circulatory assist for small children is associated with little success, particularly in patients with single-ventricle physiology.[158] The fact that human infants rarely have anti-TKO pig antibodies (*Figure 2C and D*),[39] and the observation that the administration of an anti-CD154 mAb prevents the development of even natural anti-pig antibodies (as well of elicited antibodies)[159] strongly suggests that a cardiac xenotransplant in this age group would likely be life-supporting until a suitable allograft became available.

An important question is whether, if the recipient becomes sensitized to the pig organ graft, this will be detrimental to the outcome of subsequent cardiac allotransplantation. The current, and increasing, evidence is that sensitization to a pig xenograft will not be detrimental to a subsequent allograft.[160–162] This is in contrast to the evidence that indicates that prior allosensitization may be detrimental to a subsequent allograft whereas it has been clearly documented that the anti-HLA alloantibodies do not cross-react with pig antigens.[162,163]

Attempting cardiac XTx as a bridge in paediatric patients who are at high risk of death while awaiting allotransplantation would seem ethically justified, with little risk of causing (additional) sensitization to alloantigens.[160–162,164]

Finally, yet importantly, how should such studies be planned? In the beginning, only a few patients would be included in a pivotal or pilot study. Assuming their successful long-term outcome, the up-scaling of a herd of safe source pigs will then be next (*Figure 6*). One big central production unit (or farm) per continent would probably be enough to serve the needy patients: with the porcine hearts perfused, they will be transported to the various cardio-surgical clinics located all over, e.g. Europe. Regulatory authorities will demand strict biobanking and data collection, an ideal opportunity to test their success vs. implantation of mechanical assist devices.

7. Safety of XTx (potential infectious complications)

The microbiological and virologic safety profile of porcine xenotransplants is very high since GM donor pigs can and must be maintained in designated pathogen-free (DPF) barrier facilities ensuring the absence of zoonotic pathogens (reviewed previously[165,166]). Successful concepts for the design of DPF facilities are in place (e.g.[167]). In addition, highly sensitive and specific assays have been established for specific pathogens which must be absent from the donor pigs.[168] Some of them, e.g. the porcine cytomegalovirus, had a significant negative effect on cardiac xenograft survival in pre-clinical transplantation experiments[169] and may have contributed to the Maryland heart xenograft recipient's demise.[29] It is thus mandatory to use strictly DPF donor pigs and confirm the absence of porcine cytomegalovirus using sensitive PCR assays[170] and serological methods. In addition to the targeted screening approach, next-generation sequencing offers the opportunity to screen donor pigs in a holistic manner, potentially even detecting currently unknown infectious agents.[171]

Of special importance are the porcine endogenous retroviruses (PERVs). PERVs are integrated in the genome of pigs: PERV-A and PERV-B are present in the genome of all pigs, whereas PERV-C is in the

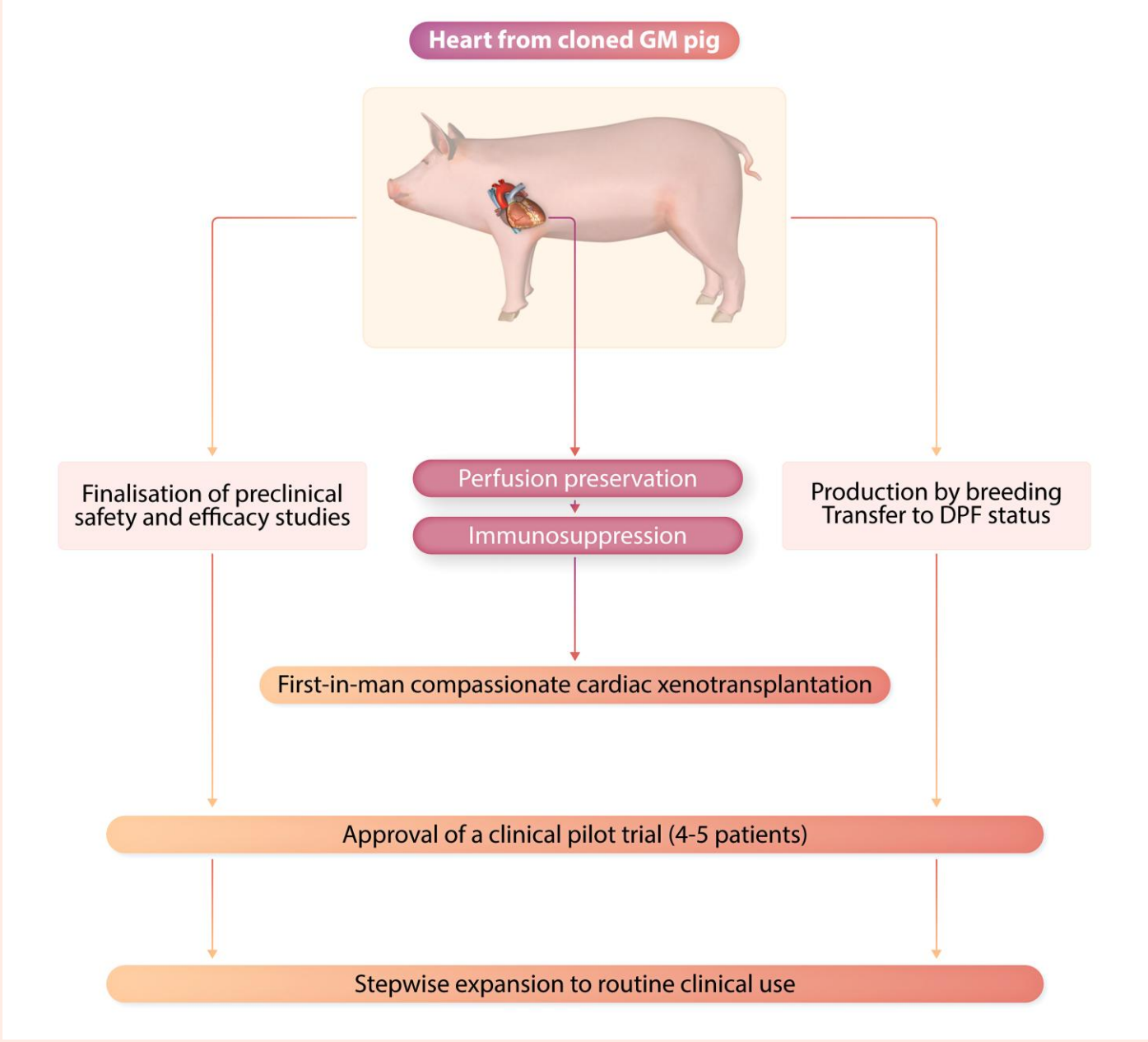


Figure 6 Stepwise clinical translation of cardiac xenotransplantation. After the first compassionate use of a 10× GM pig heart for a patient, ongoing pre-clinical studies need to be finalized and the donor pigs need to be transferred to a designated pathogen-free (DPF) status to get approval for a clinical pilot trial. After successful completion, stepwise expansion to routine clinical use can be envisaged.

genome of most, but not all, pigs. PERV-A and PERV-B are polytropic and can infect human cells *in vitro*, whereas PERV-C is ecotropic and infects only pig cells.[172] Recombinants between PERV-A and PERV-C infect human cells *in vitro* and are characterized by higher replication rates compared with PERV-A.[173,174] PERV-A, PERV-B, and PERV-A/C have been shown to infect mostly human tumour and immortalized cells, but rarely primary cells. To date, PERV transmission has not been detected in numerous XTx pre-clinical trials in NHPs and other species, in *in vivo* infection experiments in different species,[175] nor in the clinical XTx of pig islet cells in diabetic patients.[176,177]

Several strategies have been proposed to prevent PERV transmission: (i) selection of pigs with low expression of PERV and therefore a low probability to release infectious particles; (ii) selection of PERV-C-negative animals to prevent PERV-A/C recombination; (iii) vaccination of the recipient before transplantation; (iv) use of anti-retroviral drugs; and (v) inhibition of PERV expression by RNA interference.[172] In addition, pigs with inactivated PERVs have been generated using CRISPR/Cas9 technology.[74] Since this strategy may be associated with several problems, such as severe off-target effects[178,179] and reduced viability of the pigs,[74] and to date no PERV transmission has been observed in pre-clinical and clinical XTx trials,[176,180] the question arises whether this strategy is required for safe XTx.[181]

8. Ethical considerations and regulatory aspects

The ethical aspects of XTx have been discussed extensively in several reviews and commentaries.[182–186] Many are similar to those raised regarding

allotransplantation, and others relate to animal welfare or the biotechnology industry. However, the significant benefits of XTx must not be overlooked, e.g. negating the illegal trade in organs from living human donors, and eliminating the (small) risk associated with the excision of kidneys from healthy altruistic living donors. The question of whether the recipient of a pig organ, who will need to be monitored for potential pig-related complications throughout life, can withdraw from a clinical trial has been raised.

There will always be those who object to the use of animals, but the fact that in the USA alone more than 100 million pigs are slaughtered each year for food reduces the concern for using pigs for these life-saving procedures. Organ-source pigs will be housed under ideal conditions and will be euthanized under anaesthesia after the surgical removal of the organs. This will be much more humane than the methods of killing pigs in industrial farming facilities and will ensure that the detrimental effects of brain death are not present in the organs.

In summary, there are no general ethical or religious objections against clinical XTx trial as long as effective concepts for informed consent and proper regulations are in place.[136,187]

The regulatory framework for XTx in the United States has been summarized in a recent letter.[188] In brief, the US Food and Drug Administration (FDA) has a well-established paradigm for the regulation of XTx products. The Center for Veterinary Medicine (CVM) is responsible for assessing intentional genomic alterations (IGAs) in the source pigs. The Center for Biologics Evaluation and Research (CBER) is responsible for ensuring the safety and effectiveness of biologics, including XTx products (defined as 'the transplantation, implantation, or infusion into a human recipient of either live cells, tissues, or organs from a non-human animal source; or human body fluids, cells, tissues, or organs that have had *ex vivo* contact with live non-human animal cells, tissues, or organs'). CVM and CBER collaborate on their assessments of animals used for XTx. Marketing of an IGA in an animal, including its use as a source of organs, tissues, or fluids in XTx, requires that the CVM approves an application for the IGA. Multiple IGAs in source pigs for XTx are considered under a single application for approval. Clinical trials require the submission of an Investigational New Drug (IND) application. Introduction of a XTx product into interstate commerce requires an approved Biologics License Application (BLA). For the product to receive approval, the clinical trial data submitted to the FDA must demonstrate the safety and effectiveness of its intended use.

In the European Union (EU), ordinances and guidelines on Advanced Therapy Medicinal Products (ATMP; EC/1394/2007), pharmacovigilance (2010/84/EU and EC/1235/2010) as well as clinical trials (EU/536/2014) have created a regulatory framework that is relevant for XTx. Principally, the existing regulatory framework for XTx is suitable to protect the fundamental rights of both human participants and animal subjects. In addition, in the 27 EU member states, national laws such as on medicinal products, genetic engineering, and protection against infection may be implemented.

The ATMP regulation on XTx has some limitations since animal organs are not explicitly mentioned, even though they may be derived from GM animals and thus be substantially manipulated compared with organs derived from wild-type animals.

In the guideline, the definition of somatic cell therapeutics as well as the definition of tissue-engineered products of animal origin is based on tissues or cells but excludes organs. Nonetheless, organs derived from GM animals contain tissues and cells. For specificity, the European Medicines Agency (EMA) has published the guideline on Xenogeneic Cell-Based Medicinal Products (EMEA/CHMP/CPWP/83508/2009).

Central elements of ATMP regulation EC/1394/2007 include (i) designation of the EMA to authorize or grant marketing designation for XTx products within the EU, (ii) requirement for xenograft traceability from creation through clinical use and ultimate disposition, and (iii) hospital exemption for medicinal products that are not routinely prepared.

In the EU, regulatory paths to yield marketing authorizations for medicinal products, including ATMP, are based on data that cover product quality, non-clinical assessment (i.e. pre-clinical trials), and clinical trials. Data must be summarized by the applicant, often the pharmaceutical entrepreneur working in partnership with clinical investigators and their medical institution(s), in dossiers including a standardized set of Common Technical Documents (CTD), which are expected to show consistent data on product quality, safety, and efficacy. EMA offers scientific recommendation on the classification of ATMP according to Article 17, EC/1394/2007.

Most likely, regulatory requirements in the EU and in the member states will be adapted according to the scientific and technical progress in XTx.

9. What do we predict the future of cardiac XTx will be during the next 5–10 years?

Allografts will always be preferable for humans with advanced (end-stage, terminal) myocardial disease after all other conventional treatments fail, like medical therapies and electrophysiology procedures. Methods that expand the existing donor pool will be needed, like heart procurements after circulatory death[189] or/and improved graft function using innovative perfusion techniques.[127] Unfortunately, even then there will be not enough organs. After decades of thorough research, a cardiac XTx is now a realistic option and the approval given by the US FDA to the University of Maryland group for the performance of a single pig heart transplant in January 2022 is greatly encouraging.[28] The FDA recognized the need for XTx and accepted that (i) pigs with multiple GMs would be required; (ii) cloned pigs could be used for the initial studies; (iii) complete inactivation of PERVs was not required; and (iv) a co-stimulation pathway blocking agent was administered even though it was not yet approved by the FDA.

On the basis of these observations, we suggest that bridging with a pig heart xenograft will be introduced into the clinic within the next year or two, possibly initially again on an individual compassionate basis, but preferably as part of a formal clinical trial. We expect that trials in both infant and adult patients will be approved. With successful longer-term experience, we predict that cardiac XTx as destination therapy will soon be an accepted treatment form.

In regard to offering a treatment option for patients with terminal heart disease, we firmly anticipate that the advances that will be made in the field of XTx during the next decade will far surpass those that can be anticipated in the development of mechanical devices, stem cell technology, and regenerative medicine.

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