

## Minireview

# Xenotransplantation: Infectious Risk Revisited

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**Xenotransplantation is a possible solution for the shortage of tissues for human transplantation. Multiple hurdles exist to clinical xenotransplantation, including immunologic barriers, metabolic differences between pigs – the source species most commonly considered – and humans, and ethical concerns. Since clinical trials were first proposed almost 10 years ago, the degree of risk for infection transmitted from the xenograft donor to the recipient has been extensively investigated. A number of potential viral pathogens have been identified including porcine endogenous retrovirus (PERV), porcine cytomegalovirus (PCMV), and porcine lymphotropic herpesvirus (PLHV). Sensitive diagnostic assays have been developed for each virus. Human-tropic PERV are exogenous recombinants between PERV-A and PERV-C sequences and are present in only a subset of swine. Porcine cytomegalovirus can be excluded from herds of source animals by early weaning of piglets. In contrast, the risks associated with PLHV remain undefined. Microbiologic studies and assays for potential xenogeneic pathogens have furthered understanding of risks associated with xenotransplantation. Thus far, clinical xenotransplantation of pig tissues has not resulted in transmission of viral infection to humans; significant risks for disease transmission from swine to humans have not been confirmed. If immunologic hurdles can be overcome, it is reasonable to initiate carefully monitored clinical trials.**

**Key words:** Clinical trials, cytomegalovirus, ethics, herpesvirus, infection, pig, porcine endogenous retrovirus, swine, transplantation, viral recombination, xenotransplantation

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## Introduction

Many people are awaiting organ or cell transplantation worldwide. Others who might benefit from organ transplantation are either not yet 'sick enough' to be placed on

official waiting lists, or suffer from diseases (diabetes, cirrhosis) that might be treated with organ, islet or cellular transplants if the supply of tissues were increased dramatically. In 1907 Alexis Carrel suggested that the future of transplantation for the treatment of organ failure was 'heterotransplantation'. This transplantation of cells, tissues, or vascularized organs from nonhuman species into humans, now termed xenotransplantation, would have the theoretical advantage of providing an unlimited supply of transplantable organs as either permanent organ replacements or as 'bridges' to the availability of human-derived organs (1–5). In addition, these organs might be resistant to infection by common human pathogens such as hepatitis C virus or HIV.

Unfortunately, multiple barriers currently exist to the broad application of xenotransplantation, including immunologically mediated graft rejection of xenogeneic tissues, possible metabolic or molecular incompatibilities between donor organs and humans, ethical concerns, and the risk of infections that might be transmitted from the donor species to the human recipient and to the general human population. The likelihood of clinical success of xenotransplantation was initially enhanced by the development of genetically modified swine which express human complement regulatory proteins. More recently, pigs have been produced that have disrupted genes for the enzyme alpha-1,3-galactosyltransferase; these swine are unable to express  $\alpha-1,3$ -gal (Gal) sugars on their cells, which is the major endothelial antigen targeted by hyperacute, humoral graft rejection. The combination of immune suppression, use of organs across histocompatibility barriers, and the absence of pre-existing immunity in the recipient to animal-derived organisms, render the host more susceptible to infection. Consequently, infection is likely to remain a significant barrier to xenotransplantation even as immune barriers are breached. Indeed, public attention regarding xenotransplantation has focused less on these immunologic hurdles than on the possible spread of infection from nonhuman species to human xenograft recipients and into the community at large (6–11). This theoretical risk merits reconsideration in light of data assembled over the past 10 years regarding the degree of infectious risk posed by xenotransplantation.

## Background

Experience with immunocompromised patients suggests that novel pathogens may emerge as a cause of infection, including organisms not normally associated with

human disease (7,12,13). Transplantation poses a unique epidemiologic hazard due to the efficiency of the transmission of pathogens, particularly viruses, with the grafts. The term 'xenosis' (also 'direct zoonosis' or 'xenozoonosis') was coined to reflect the unique epidemiology of infection resulting from organisms carried by xenogeneic tissues. A number of factors may increase the risk of infection in xenotransplantation:

- The xenograft serves as a permissive reservoir in which donor organisms bypass host defenses without a need for a 'vector' to achieve disease transmission;
- lack of knowledge about the behavior of organisms from the donor species in immunosuppressed humans;
- inability to recognize novel clinical syndromes resulting from infection with such pathogens;
- lack of clinical laboratory assays for organisms from nonhuman species;
- donor-derived organisms may not cause disease in the native host species but may cause disease in a new host ('xenotropic organisms'), or may acquire new characteristics (genetic recombination or mutation) (14–17); and
- donor-recipient incompatibility of major histocompatibility (MHC) antigens may reduce the efficacy of the host's immune response to infection within the xenograft.

Swine are most often considered as the source species for xenografts. While nonhuman primates are closer immunologically to humans, ethical issues, the risk of transmission of viruses known to be infectious for humans, poor size matches, and the expense and difficulty in breeding have excluded this option. Concerns raised regarding the infectious risks associated with the use of nonhuman primates as organ donors for humans have led the U.S. Food and Drug Administration to preclude the use of these species as organ donors for humans. Swine, while immunologically dissimilar, are easier to breed, can be good size matches for humans, and have been genetically engineered to express or suppress specific genes relevant to transplantation.

Initial microbiological concern focused on pig-derived bacteria or parasites. Organisms such as *Streptococcus suis* could not be easily characterized by most clinical microbiology laboratories. However, the ability to exclude extracellular organisms and bacteria from herds of swine destined for use as organ donors has focused attention on potential viral pathogens. Theoretical concerns regarding transmission of novel pathogens from pigs to humans gained substance with the description of a family of porcine endogenous retroviruses (PERVs) and the demonstration that this family of viruses is capable of infecting human cells *in vitro* (18–20). Characterization of other potential pathogens from swine will provide evidence-based approaches to infection prevention in xenotransplantation (21–25).

## Barriers to Successful Clinical Xenotransplantation

The main hurdles to successful xenotransplantation have been immunologic. Hyperacute graft rejection (HAR) is the effect of preformed ('natural') antibodies present in humans and other Old World primates against a ubiquitous Gal epitope expressed on (porcine) vascular endothelial cells (2,4,5,26–32). With HAR, the binding of antibody with complement deposition results in the rapid death of vascularized xenografts. Strategies to prevent hyperacute rejection have included depletion of anti-Gal antibodies and genetic engineering of swine to express human complement regulatory proteins on porcine xenografts to decrease complement deposition and tissue injury. However, some of these complement regulatory proteins also serve as cellular receptors for human pathogens (e.g. CD46 and measles) to which swine are not naturally susceptible. Consequently, it is possible that human measles virus might be given an opportunity to grow in transgenic porcine cells and have the capacity to infect either the transgenic swine, or transgenic xenografts in human hosts (33). It is also notable that natural antibodies may provide an immunological defense against human infection by retroviruses, parasites and other common organisms carrying Gal epitopes. Thus, depletion of anti-Gal antibodies may, theoretically, allow transmission of porcine viruses (carrying the Gal epitope) from a porcine xenograft to the host. However, the role of such antibodies *in vivo* remains unknown.

Recently, a number of 'knock-out swine' have been born with disrupted genes for alpha-1,3-galactosyltransferase, and lack the target sugar for HAR (34). Xenografts from these animals survive longer than unmodified grafts (D. H. Sachs, personal communication). The use of such knock-out organs avoids the need to deplete natural antibodies in the recipient and the theoretical side-effects noted earlier. It should be noted that in the absence of Gal sugars on the cell membranes of knock-out porcine xenografts, enveloped porcine viruses produced in the xenograft will lack this epitope and that the protection provided by natural antibodies will be lost.

Similarly, 'tolerance induction' (antigen-specific immunologic unresponsiveness) and a variety of immunosuppressive regimens are under study to prevent chronic, cellular rejection which occurs over days to weeks after pig-to-nonhuman-primate transplants. The infectious consequences of such manipulations remain to be determined (e.g. Does the tolerant host also become tolerant of latent or endogenous infections carried by graft tissues, facilitating virus transmission?).

## Identifying Potential Human pathogens

While there are many organisms that are xenotropic for humans, it has been proposed that only a subset pose a

**Table 1:** Categories of potential pathogens resulting from xenotransplantation

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1	'Traditional zoonosis': Well characterized clinical syndromes of humans (e.g. <i>T. gondii</i> ) – specific diagnostic assays generally available
2	'Species-specific': Organisms incapable of causing infection outside the xenograft (e.g. porcine CMV) – some tests available, few standardized assays available for humans
3	'Potential pathogens': Organisms of broad 'host range' which <i>may</i> spread beyond the xenograft (e.g. adenovirus) – few specific diagnostic assays available
4	'Unknown' pathogens: Organisms not known to be human pathogens and for which clinical syndromes and microbiologic assays are not available

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- New virulence characteristics within a host; i.e. xenotropic organisms
- Viral recombinants resulting from intentional genetic modification of donor diseases resulting from multiple simultaneous infections (e.g. lymphosis of cattle resulting from *Babesia* and viral coinfection)

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particular threat to the immunocompromised human host (Table 1). However, without confirmation in animal models or in humans, such predictions remain merely educated guesses based on experience in immunocompromised humans. For example, relatively benign organisms may increase in virulence with passage in a new host (evolutionary adaptation) or may cause no disease in their native species while causing disease in xenograft recipients. Accordingly, lists have been generated to guide the breeding of source animals for xenotransplantation including organisms thought likely to cause disease in xenograft recipients based on known ability to infect humans (*Toxoplasma gondii*), similarity to organisms commonly causing infection in transplant recipients (e.g. porcine cytomegalovirus), or organisms with a high predilection for recombination (e.g. parvovirus). In addition there may be organ-specific exclusion lists (e.g. *Mycoplasma* sp. or influenza virus in lung donors). Such lists, while inexact in the absence of clinical experience, serve a variety of purposes in the progress of xenotransplantation. These include:

- 1 Organisms thought to pose an unacceptable risk to the recipient can be bred out of a donor herd prospectively ('designated pathogen-free' or DPF) (7,9);
- 2 Microbiologic assays for these organisms can be developed for clinical use;
- 3 Studies in preclinical xenograft models may clarify the biology of these organisms; and
- 4 Prophylactic strategies can be developed for organisms not 'removed' from donors.

Organism exclusion lists will vary with the donor species and the use intended for the xenograft. Accordingly, such microbiological standards must be 'dynamic' – rigorously tested and subject to revision based on experimental and clinical data. Standards for testing must also reflect the evolution of testing strategies (e.g. new quantitative molecular assays) and adjusted for differences in immunosuppressive regimens and epidemiology. 'Archiving' of tissue and serum samples from donor animals and recipients has been mandated by FDA guidelines for future use in tracking unsuspected or novel pathogens in clinical trials of xenotransplantation.

### **Retroviruses**

Exogenous retroviruses (HTLV-1, HTLV-2, and HIV) have been transmitted with human tissues during organ and cell transplantation and are associated with active infection. The course of accidentally transmitted infection resulting from HIV-1 can be accelerated in transplant recipients, manifesting disease (AIDS) within 6 months (35). Concern about retroviral transmission in xenotransplantation relates to the potential for 'silent' transmission, i.e. an unapparent infection which may cause altered gene regulation, oncogenesis, or recombination. The activation of latent virus and the development of clinical manifestations, if any, may be delayed for more than a decade. Pigs do not possess exogenous viruses equivalent to HTLV or HIV. However, endogenous retroviruses (part of the germ line DNA) have been demonstrated in all species studied to date. Endogenous retroviruses that are infectious for human cells *in vitro* have been detected in many mammalian species including baboons (BaEV), cats (RD114), mice (murine ERV), and pigs (PERV). Although the pig genome contains many sequences closely related to mouse mammary tumor virus (B-type), murine leukemia virus (C-type), and Mason-Pfizer monkey virus (D-type) retrovirus sequences, only three closely related C-type PERV (PERV A, B, C) have been identified in swine that possess infectious potential (36–39). Two of these, PERV-A and -B, can infect human and pig cells *in vitro* (20). The third subgroup, PERV-C, infects porcine cells only (20). Infectious forms of the remaining PERV families have not been isolated and are unlikely to encode infectious virus owing to disruptions in open-reading frames (ORFs) (36). Pig ERV mRNAs are expressed in all pig tissues and in all breeds of swine tested to date, and expression can be amplified by stimulation of swine peripheral blood lymphocytes *in vitro*. There is variation between tissues in terms of the size and amount of PERV mRNA transcripts, consistent with *in vivo* recombination and/or processing (18).

Porcine endogenous retrovirus-A and -B infect several human cell lines and primary cell cultures (20,38–46). Swine can be classified according to whether their peripheral blood mononuclear cells (PBMCs) either do or do not transmit PERV to human cells *in vitro*. Such animals are termed either 'transmitters' or 'nontransmitters'. High-titer

## Fishman and Patience

human-tropic PERVs isolated from 'transmitting' animals are recombinants between PERV-A and PERV-C sequences (38,47). Although the site of recombination varies, viral sequences are derived from the recombination of PERV-A elements with the post-VRA (envelope) region of PERV-C. Therefore, although PERV-C is not capable of infecting human cells, it appears to be an essential component of high-titer human-tropic PERV from these swine and may be important in the assessment of infectious risk associated with xenotransplantation. The source of such recombinants *in vivo* is unknown; analysis of the germ-line DNA of transmitting animals has not identified the presence of such recombinant viruses (48). Thus, swine with incomplete genomic provirus (i.e. Porcine endogenous retrovirus A without a complete *env* gene) might be able to generate infectious recombinant viruses in the presence of infectious PERV-C. No evidence of infection has been demonstrated of human cells *in vivo* and no disease resulting from this family of viruses has been described in swine or humans to date (49–54). Porcine endogenous retrovirus appears to be susceptible to currently available antiviral agents (55). Some data suggest that primary cell lines of primates (baboons, gorilla, and macaques) can be infected by PERV-A, -B, and possibly -C, which enhances the value of preclinical studies in primates (56). However, other studies question the value of these models, as the infection of nonhuman primate tissues is often abortive. Whether humans are equally nonpermissive remains to be determined.

### Herpesviruses

Activation of latent herpesvirus infection during periods of intensified immune suppression or immune dysfunction and by immune reactivity to grafts (rejection) is an important problem in human allotransplantation (12). Comparable viruses exist in swine but tend to be species-specific, and would be expected to cause infection only in tissues derived from the usual host species for each viral strain.

Most importance is placed on cytomegalovirus (CMV), which causes invasive tissue disease, fever and neutropenia and immune modulation which can contribute to the risk for secondary infections, graft rejection, and lymphoma. Extensive molecular screening identified three families of herpesviruses in swine: porcine CMV, and porcine lymphotropic virus-1 and -2 (PLHV-1, -2). Replication of porcine cytomegalovirus (PCMV) is enhanced by intensive immune suppression in pig-to-primate models of xenotransplantation (21,22). Porcine cytomegalovirus infection causes tissue-invasive infection in porcine xenografts in baboon hosts and contributes to endothelial injury and consumptive coagulopathy (CC) in some animals (57). Based on molecular and histological evaluations, PCMV does not appear to cause invasive disease in tissues of baboons that have received porcine xenografts (21). It is possible to exclude PCMV from herds of swine by early weaning of newborns (58). The absence

of PCMV improves graft survival and reduces coagulopathy in pig-to-baboon xenotransplantation. Recent data suggest that porcine CMV has reduced susceptibility to ganciclovir, foscarnet, and cidofovir compared with human strains (59).

Two novel families of gamma-herpesviruses have been identified in swine by amplification of short DNA polymerase sequences from pigs, porcine lymphotropic herpesvirus-1, -2, and -3 (PLHV-1, -2, -3; 60). Porcine lymphotropic herpesvirus-1 is associated with a syndrome of lymphoid proliferation in swine undergoing experimental allogeneic hematopoietic stem-cell transplantation. This syndrome has characteristics similar to post-transplantation lymphoproliferative disease (PTLD) (5,23,24,60). Based on sequence analysis, this virus has some genetic homology with known sequences of lymphocryptovirus (EBV) and the rhadinoviruses (HHV8; 24). The role of this virus in the pathogenesis of PTLD is under investigation. In porcine allogeneic hematopoietic stem-cell transplantation, the risk of PTLD in swine is related to the overall intensity of immune suppression, the MHC disparity between donor and host, the degree of T-cell depletion, and the PLHV activation that precedes B-cell proliferation. The role of PLHV-2 and -3 are unknown. Unlike PCMV, PLHV-1 is not removed from source animals by early weaning of newborns (61).

### Other potential pathogens

A variety of potential human pathogens have been described in swine. These include porcine circovirus types 1 and 2, porcine reproductive and respiratory syndrome virus, porcine encephalomyocarditis virus, swine influenza viruses, African swine fever virus, hepatitis E-like virus, pseudorabies virus, parvovirus, and polyomaviruses of swine. None has yet been associated with human disease.

Exposures of humans to products derived from pigs and other nonhuman species have had no demonstrable adverse effects on individuals or the general population. Cesarean-derived porcine fetal tissues intended for human xenotransplantation carried antibodies to *Leptospira interrogans* and *Aspergillus fumigatus* (62–65). Bacterial, viral, fungal, and parasitic evaluations of transplanted cell preparations were negative and no infections were reported when transplanted into humans. Transplantation of porcine fetal brain cells for the treatment of refractory Parkinson's disease and intractable seizures with minimal immune suppression have been achieved without infectious complications to date (66,67), although only limited numbers of clinical trials have been performed.

Recent human epidemics of viral infection have been traced to animal-derived strains that have been adapted to human hosts, heightening concerns about possible animal-to-human disease transmission with xenotransplantation. These include hantavirus (mice), SARS (severe acute

respiratory syndrome owing to a new coronavirus possibly associated with civets), BSE (bovine spongiform encephalopathy), and HIV (human immunodeficiency virus thought to evolve from primate viruses). In each case, the epidemiology was defined after the recognition of a new clinical syndrome and the development of new, rapid, molecular assays for the causative agent [discussed in (68)]. As an example, sporadic cases of zoonotic human infection with swine influenza A viruses have been detected in the United States, Europe, Asia, and New Zealand since 1976, most with direct evidence of exposure to swine (69,70). Influenza A viruses circulate in many animal hosts that may serve as a reservoir for human disease. In most cases, these viruses are not adapted for human infection and cannot cause human disease. As a result, the number of individuals affected is small compared with the number involved in pig farming (71). Pigs may also serve as hosts for the adaptation of avian respiratory viruses to human hosts. Thus, human, porcine, and avian viruses may undergo genetic reassortment in swine to produce novel strains of pandemic potential (72–75). It is possible that infected lung xenografts might, for example, provide the 'Petri dish' for recombination between porcine and human influenza viruses, particularly in the immunocompromised host. This theoretical concern can be addressed via the breeding of herds of source animals in isolation from human respiratory viruses from animal caretakers and adventitious birds and rodents. Given highly sensitive molecular assays, it is feasible to assure that pigs used as source animals for xenografts, particularly lungs, be screened for influenza viruses.

Similarly, hepatitis E virus (HEV) is a major cause of viral hepatitis in the developing world and has recently been detected in swine in North America and Asia. While spread of HEV to humans from pigs has not been demonstrated, the viruses found in swine are closely related genetically to those causing human disease, suggesting that pigs may serve as a reservoir of infection (76,77). Clearly, this would be merit special screening of animals to be used as a source of porcine hepatic grafts.

#### **Routine monitoring for xenogeneic infection**

In xenograft recipients the risks of infection and rejection will necessitate life-long monitoring. In addition to the recipients, intimate contacts of the recipients might also be at increased risk for xenogeneic infection. In individuals receiving organ transplants, infection, graft rejection, malignancy, and other etiologies of organ dysfunction are often indistinguishable on clinical grounds. In xenograft recipients, these signs may be manifestations of common, community-acquired infections or latent (human) infections reactivated in the recipient. Monitoring schemes have been proposed to detect known pathogens and archive specimens from source animals, and from patients, intimate contacts, and animal handlers on a routine basis for use in the event of unexplained infectious episode. These

samples may be utilized as further microbiologic assays are developed for previously unrecognized pathogens. Routine samples might be studied for the emergence of pig-derived pathogens (PERV, herpesviruses) even in the absence of clinical evidence of infection. One exciting area of research is the use of newer molecular techniques (broad-range hybridization probes or PCR primers, molecular differential display, and microarray technologies) that can be applied in xenotransplantation to detect pathogens posing a risk to xenograft recipients or their contacts.

The possibility that unexpected clinical symptoms are the result of xenogeneic infection necessitates a prepared response. The key features of such a scheme might not be dissimilar from the approach taken for allograft recipients: routine bacterial, fungal, and viral cultures performed on cells of human and pig origin before the initiation of antimicrobial therapy, PCR for PERV and herpesviruses (i.e., PCMV, HCMV, EBV, PLHV) using both sera and leukocytes, and cocultivation of peripheral blood leukocytes with human and donor-cell lines. This is followed by empiric antimicrobial therapy and hospital admission with isolation from other patients until the nature of the process is further defined. Special precautions (e.g. respiratory, secretions, neutropenia) will be dictated by the patients' clinical presentation. In the event of the recognition of a novel recombinant organism or severe infectious illness without explanation, strict isolation with HEPA filtration will be required.

#### **Infectious Risks, Surveillance, and the Search for Novel Pathogens**

The assessment of infectious risks associated with clinical xenotransplantation is central to the acceptance of this technology and to optimal patient care. Thus, it is important to investigate potential pathogens both in pre-clinical models and in xenograft recipients. Significant progress has been made in defining risks resulting from known pathogens. The identification of PERV and other viruses have allowed the development of sensitive assays for these agents and strategies for exclusion of these pathogens from xenograft donors. There may be additional human pathogens in swine not yet identified. Most should be identifiable in preclinical models while others may appear only in clinical trials. Thus, it is essential that investigators share clinical and preclinical data that suggest the presence of unusual infectious events in xenotransplantation. Without a commitment to sharing epidemiologic data, the 'occasional' infectious event or 'novel syndrome' is likely to remain unrecognized – below the 'epidemiological radar screen'. Approaches to the sharing of data internationally have been addressed (see the 'Consultation on Xenotransplantation Surveillance' sponsored by the OECD, WHO, and Health Canada, <http://www.oecd.org>). These emphasize the need for shared definitions for xenogeneic infectious disease events (case definition, laboratory assays,

## Fishman and Patience

and specific organisms) and international cooperation in reporting, recording, and response to adverse events associated with xenotransplantation.

## Benefits of Xenotransplantation

Concerns regarding potential infectious risks of xenotransplantation have generally overwhelmed discussions about the potential benefits of this technology. However, some of the major infectious hazards currently confronted in human allotransplantation can be addressed via elective xenotransplantation should this become practical for broad clinical application. Some of the unique benefits of xenotransplantation are derived from:

- *Careful microbiological screening* of the animals used for xenotransplantation (as compared with limited screening of human tissues performed before allotransplantation);
- Potential *resistance of the xenogeneic tissues to infection by human pathogens* including HIV (1,2), HTLV, hepatitis viruses, and herpes viruses (including human CMV) (75). For example, porcine cytomegalovirus does not appear to infect baboon tissues *in vivo* (21). This 'species specificity' may reflect the absence of receptors or of cellular 'machinery' necessary for viral replication in human cells;
- *Limited duration of exogenous immune suppression* is a component of many proposed xenotransplantation protocols, which include immunologic tolerance induction, which may reduce the risk for opportunistic infections; and
- *Patients can receive their transplants at the time of greatest clinical need*, reducing the duration of pre-transplant hospitalization and exposure to nosocomial pathogens. This also avoids the prolonged hospitalizations and colonization common in many deceased donors.

## Moving to Clinical Trials

Significant advances have been made in the microbiology of xenotransplantation. Concerns regarding xenotransplantation were raised by these authors and others when clinical trials were first proposed and focused largely on those pathogens that could not be 'removed' by selective breeding – the endogenous retroviruses and possibly herpes viruses of swine. Data generated over the past 10 years suggest that the risks of human infection resulting from xenotransplantation are manageable. More so, the risk of the spread of infection to contacts of the xenograft recipient appears to be low. Careful screening and early weaning of donor swine can exclude many potential human pathogens in isolated herds with routine microbial surveillance. Unfortunately, the risk of infection will never be zero. Further studies in swine will elucidate pathogenic mecha-

nisms (e.g. retroviral recombination and the pathogenesis of post-transplant lymphomas). Current data suggest, however, that consideration can now be reasonably given to research into other barriers to clinical xenotransplantation (immune and metabolic) as a prelude to clinical trials.

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