Provision of safe donor pigs for xenotransplantation

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Für meine liebe Familie

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Abbreviations

αGal galactose α-1,3 galactose

AAALAC Association for Assessment and Accreditation of Laboratory Animal Care

AHXR acute humoral xenograft rejection

AIDS acquired immune deficiency syndrome

ASLV avian sarcoma/leukosis virus

ATMP Advanced Therapy Medicinal Product

BaPAR-2 baboon PERV-A receptor 2

bp base pair

BHK-21 baby hamster kidney cell line

°C degree Celsius

Cas CRISPR-associated

CiMM Center for innovative Medical Models

CMAH cytidine-monophosphate-N-acetyl-neuraminic acid hydroxylase

CPB cardiopulmonary-bypass

CRC TRR 127 Transregional Collaborative Research Center 127 (short "SFB" for the German "Sonderforschungsbereich")

CRISPR clustered regularly interspaced short palindromic repeats

CXTx cardiac xenotransplantation

DPF designated pathogen- free

DMD Duchenne muscular dystrophy

DNA deoxyribonucleic acid

e.g. exempli gratia

ECM decellularized extracellular matrix

EDTA	Ethylendiamintetraazetat
EMA	European Medicines Agency
env	envelope
ERV	endogenous retrovirus
ET	embryo transfer
etc.	et cetera
EtOH	Ethanol
FASS	Federation of Animal Science Societies
FCS	fetal calf serum
FDA	Food and Drug Administration
FELASA	Federation of European Laboratory Animal Science Associations
FeLV	feline leukemia virus
GaLV	gibbon ape leukemia virus
GGTA1	α-1,3-galactosyltransferase
gm	genetically-modified
GMP	good manufacturing practice
GTKO	GGTA1 knock-out
GV-SOLAS	Gesellschaft für Versuchstierkunde / Society of Laboratory Animal Science
H_2O	water
HAR	hyperacute xenograft rejection
hCD46	human membrane cofactor protein CD46
hCD55	human decay acceleration factor CD55
hCD59	human membrane inhibitor of reactive lysis CD59
HCV	hepatitis C virus

hCXTx heterotopic cardiac xenotransplantation

HEV Hepatitis E virus

HEK human embryonic kidney

HIV human immunodeficiency virus

HLA human leucocyte antigens

hTBM human thrombomodulin

HuPAR-1 human PERV-A receptor 1

HuPAR-2 human PERV-A receptor 2

HUVECs human umbilical vein endothelial cells

ICM inner cell mass

ICTV International Committee on Taxonomy of Viruses

IHC immunohistochemistry

iPSCs induced pluripotent stem cells

ISHLT International Society for Heart and Lung Transplantation

IVF *in vitro* fertilization

IXA International Xenotransplantation Association

KoRV koala retrovirus

LTR long terminal repeat

LVG Lehr- und Versuchsgut Oberschleißheim

MABB Chair for Molecular Animal Breeding and Biotechnology

MCS mechanical circulatory support

ml milliliter

MLV murine leukemia virus

mM millimolar

µl microliter

µM micromolar

MoMLV Moloney Murine Leukemia Virus

Neu5Ac N-acetyl-5-neuraminic acid

Neu5Gc N-5-glycolyl-neuraminic acid

NHDFs normal dermal human fibroblasts

NRC National Research Council

oCXTx orthotopic cardiac xenotransplantation

PBMCs peripheral blood mononuclear cells

PCMV porcine cytomegalovirus

PDNS porcine dermatitis and nephropathy syndrome

PEI Paul-Ehrlich-Institut

PERV-A porcine endogenous retrovirus A

PERV-B porcine endogenous retrovirus B

PERV-C porcine endogenous retrovirus C

PERVs porcine endogenous retroviruses

PCV1 porcine circovirus type 1

PCV2 porcine circovirus type 2

PCV3 porcine circovirus 3

PHS U.S. Public Health Service

PoPAR porcine PERV-A receptor

PSC pluripotent stem cell

RNA ribonucleic acid

rpm rounds per minute

RT	reverse transcriptase
SARS	severe acute respiratory syndrome
SCNT	somatic cell nuclear transfer
SOPs	standard operation procedures
SPF	specific pathogen free
TAH	total artificial heart
TALENs	transcription activator-like effector nucleases
TE	trophectoderm
THBD	thrombomodulin
TLA	Targeted Locus Amplification
TTS	The Transplantation Society
UDP-Gal	uridine-diphosphate galactose
UV	ultraviolet
WHO	World Health Organization
ZFNs	zinc finger nucleases

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1 Introduction

Cardiovascular diseases were the most common cause of death in the year 2017, since 37,0 % of all deaths in Germany were due to chronic ischemic heart disease and acute myocardial infarction (heart attack) (DESTATIS, 2019). The only cure for patients with cardiac end stage disease is heart transplantation, but the number of available human donor organs is anywhere near from the clinical need (LUND et al., 2017; EUROTRANSPLANT, 2018; ORGANTRANSPLANTATION, 2019). The demographic change and the advanced possibilities of modern society and medicine have led to an increase of elderly persons, who are even more susceptible to cardiovascular diseases. Moreover, not only the increase of elderly, but also the growth of diabetic and overweight people adds to the expanding demand for donor organs.

Heart transplantation is not only performed in adults, but in pediatric patients, too, and the annual number of transplants has risen since the first transplant was performed in 1967 (ROSSANO et al., 2017). Regarding pediatric transplants, infants (under 1 year of age) have accounted for the greatest number, with more than 1600 infant transplants reported from 2004 to 2016 to the Registry of the International Society for Heart and Lung Transplantation (ISHLT). In infants, the most common diagnosis leading to transplantation was congenital heart disease (CHD). Usage of mechanical circulatory support (MCS) has continued as bridging to a transplant, with ventricular assist devices being the primary support modality. Nevertheless, in patients with CHD, especially among infants, the use of MCS was rarer. Only 12% of infants with CHD were bridged to transplant on some form of MCS (ROSSANO et al., 2017). The ongoing sensitization problem is an issue, in pediatric patients as well as in adult patients.

Here, xenotransplantation can be an alternative to the classic bridge solutions, since when pig organs are used as bridge, there should be no occurrence of this phenomenon (overviewed in COOPER et al., 2004). After all, the rising demand for deceased donor organs urges a solution with we cannot offer from one day to another, but the very promising results in the field of xenotransplantation over the last years promise, that this may be a feasible and perceptible alternative to allotransplantation. First steps may be the usage of pig organs as bridges to transplants with lesser complications. If this succeeds, pig organs may replace whole human organs and not only serve as a bridge. There are plenty of possible applications for xenografts, e.g. the heart, the kidney, the cornea, the skin, the lung, the small intestine, and even the liver.

To reach this goal, we have to conduct clinical trials with the safest pig organs available. In order to establish a xenotransplantation donor herd, which fits the strict requirements demanded by regulatory authorities and experts in the various fields of the xenotransplantation context, a hygiene management was established in our xenotransplantation donor herd as a first step towards safe pig organs, a clean and pathogen free herd, to provide safe donor pigs and therefore paving the way towards clinical xenotransplantation trials.

2 Literature

2.1 Introduction to xenotransplantation

2.1.1 Beginning of xenotransplantation

The idea of xenotransplantation, the transplantation of organs, tissue and cells between phylogenetically different species is not new. The very first blood transfusion between a human being, who suffered from severe fever, and a lamb took place in 1667 in Paris (reviewed in ROUX et al., 2007). The French physician Jean-Baptiste Denis performed several such xenotransfusions, but the procedure was prohibited, when one of the patients died (reviewed in DESCHAMPS et al., 2005; reviewed in ROUX et al., 2007).

The next step towards xenotransplantation to humans was the transplantation of skin in the 19th century. There were two different techniques for skin transplantation, free grafts and pedicled grafts. In the latter case, grafts were only partially detached from the donor and applied to the recipient. During engraftment donor and recipient had to be fixed together until the graft was fully vascularized on the recipient's transplantation site (GIBSON, 1955; COOPER, 1997). The fact that many donor species had appendages on the skin, e.g. feathers, wool or fur led to the use of frogs as preferred donors (GIBSON, 1955; reviewed in MOU et al., 2015).

Another significant development was the first corneal xenotransplantation performed by Richard Sharp Kissam in 1838 from pig to human. This experiment was followed by various attempts which involved alternative donor species like dog, sheep and cows, whereas the first corneal allotransplantation took place only about half a century later in 1905 (reviewed in HARA & COOPER, 2010; reviewed in COOPER et al., 2015).

A major step forward towards transplantation of whole organs was the development of blood vessel anastomosis by Nobelprize winner Alexis Carrel in 1912 (reviewed in DESCHAMPS et al., 2005; reviewed in MOU et al., 2015). A student of Alexis Carrel, Serge Voronoff, was a pioneer in endocrinotherapy. He transplanted slices of chimpanzees' testicles into the human recipient's scrotum in June 1920. Voronoff aimed for a "rejuvenation" by his surgical procedure (AUGIER et al., 1996). Remarkably, Voronoff was also the first person who struggled with the limited availability of apes, which he overcame by building so-called ape houses in

French Guinea to rear apes for exporting (reviewed in DESCHAMPS et al., 2005; reviewed in ARISTIZABAL et al., 2017). Since then primates were inevitable for xenotransplantation, either as donors or as recipients in preclinical studies.

2.1.2 Transplantation of whole organs

In the 2nd half of the 20th century replacement of organs became a valuable treatment of patients suffering from end stage organ failure. Initially, pioneers in the field followed both, allo- as well as xenotransplantation approaches.

Using the anastomosing technique introduced by Carrel, the kidney was the first solid organ to be xenotransplanted, because it is a paired organ, it is vascularized by one single artery and its function is proven by urine production (reviewed in DESCHAMPS et al., 2005). From 1963 to 1964, Keith Reemtsma performed 13 chimpanzee to human kidney xenotransplantations. There, an immunosuppression regimen with azathioprine, actinomycin C, steroids and x-radiation was included for the first time. However, none of the patients lived longer than 4-8 weeks. Only a 23-year old woman remained at good health for up to nine months until she suddenly collapsed and died (REEMTSMA et al., 1964).

About 30 years after Reemtsma's kidney xenotransplantations, in June 1992, Tom Starzl and his team did a baboon to human liver transplantation, with the 35-year old male recipient surviving for 70 days (STARZL et al., 1993).

Nearly at the same time as Reemtsma, James Hardy, performed not only the first human lung allotransplant, but was also drawn to carry out the first clinical heart allotransplantation. As his patient was in dreadful and semi-comatose state and no allograft was available, Hardy transplanted a chimpanzee heart (HARDY et al., 1963; HARDY et al., 1964). The contradictory response to this heart xenotransplantation however discouraged Hardy from further tries.

Only four years later the first successful cardiac allotransplantation was famously introduced in 1967 in Cape Town by Christiaan Barnard and his colleagues (BARNARD, 1967). Barnard also aimed at the usage of xenografts for the heterotopic cardiac xenotransplantations. He performed two, one with a baboon heart and one with a chimpanzee's heart, mainly due to shortage of human donor organs (BARNARD et al., 1977).

Barnard's work was followed by another famous clinical cardiac xenotransplantation in 1983. Leonard Bailey carried out the first cardiac xenotransplantation in a neonate, "Baby Fae". She suffered from hypoplastic left heart syndrome, received a baboon heart and survived for 20 days, as the graft experienced acute rejection (BAILEY et al., 1985; reviewed in MOU et al., 2015). Additionally, the graft was ABO-incompatible, because the blood-type O is rarely seen in baboons (DIAMOND et al., 1997). Even the innovative and highly potent immunosuppressive agent cyclosporine could not prevent cross-species rejection (reviewed in MURTHY et al., 2016). Eventually, allotransplantation of human organs became clinic routine, while xenotransplantation remained a research objective. Nonetheless, the interest in xenotransplantation remained. The main reasons are the predicted extended availability of donors and the proposed planning of transplantations. Importantly, the role of nonhuman primates changed from donors to recipients in preclinical studies and the pig, especially if genetically modified, became the preferred organ source (WEISS, 2018). The main reasons are the lower ethical concerns, well-established housing conditions and their high reproductive capacity.

The latter is highly relevant, as we are still battling shortage of deceased organ donors. In Germany for example, in 2018, 955 donors provided 3.113 organs, in contrast to 9.697 organs in demand (ORGANTRANSPLANTATION, 2019). For hearts we are facing a gap between 295 donated organs and 719 required hearts (ORGANTRANSPLANTATION, 2019). Within the Eurotransplant member states there were 619 hearts from deceased donors used, but still at the end of 2018 there were 1158 people on the active waiting lists (EUROTRANSPLANT, 2018). Alternative mechanical circulatory assist devices have greatly improved with new design and better patient survival (KIRKLIN et al., 2013; KIRKLIN et al., 2015). However, the main limitations of the mechanical assist devices are gastrointestinal bleeding, followed by heart failure and arrhythmia, infections, thrombosis and power supply limitation (HASIN et al., 2013; PATEL et al., 2014).

Furthermore, more patients would profit from a donor organ and they would profit from it at an earlier timepoint, if only there was greater availability (MOHIUDDIN et al., 2015). Before clinical application of xenotransplantation is realistic, however, solid and convincing preclinical pig-to-nonhuman primate studies are necessary.

2.1.3 Steps towards clinical trials

The first hurdle to overcome in pig-to-nonhuman primate cardiac xenotransplantation (CXTx) was hyperacute xenograft rejection (HAR), which is a complement mediated vascular injury caused by pre-formed antibodies in the recipient against galactose α-1,3galactose (αGal) epitopes on the endothelium of the graft (BUHLER et al., 1999) (reviewed in YANG & SYKES, 2007). These epitopes are synthetized by the enzyme α-1,3-galactoseyltransferase, encoded by *GGTA1*, which is functional in most of the species, including pigs, but not in Old World monkeys, apes and man (GALILI et al., 1988b; GALILI, 1993). Therefore, these species produce naturally anti- αGal antibodies, since they are exposed to αGal epitopes from gastrointestinal bacteria (GALILI et al., 1988a). The first idea to overcome HAR was to develop pigs expressing human complement regulatory proteins (MCCURRY et al., 1995; MCCURRY et al., 1996), like the membrane cofactor protein CD46 (DIAMOND et al., 2001) (hCD46), the membrane inhibitor of reactive lysis CD59 (DIAMOND et al., 1996) (hCD59) and the decay acceleration factor CD55 (LANGFORD et al., 1994) (hCD55). Additionally, pigs with various combinations of these genetic modifications were established (BYRNE et al., 1997; COWAN et al., 2000; RAMSOONDAR et al., 2003).

Rejection time of xenografts from pigs transgenic for human complement regulatory proteins varied from one week to three weeks, depending on whether immunosuppression agents were used or not (GODDARD et al., 2002; EKSER et al., 2009). Alternative approaches such as blocking the anti-Gal antibodies by *in vivo* removal (TANIGUCHI et al., 1996), intravenous infusion with carbohydrates (YE et al., 1994), conjugation of polyethylene glycol to αGal-oligosaccharides in order to achieve a prolonged action of inhibitors were tested (NAGASAKA et al., 1997). In addition, other glycoconjugates and combinations of them were compared to their ability to block anti-Gal antibody binding (BYRNE et al., 2002). Longest survival, with 139 days, was achieved by administering immunosuppression with e.g. mycophenolate mofetil, methylprednisolone, cobra venom factor and anti-CD154 mAbs on top of continuous intravenous infusion with αGal glycoconjugates (KUWAKI et al., 2004).

The ultimate solution for overcoming HAR, however, required the removal of the preformed antibody target, the αGal epitope. In 2002, the first four live heterozygous α-1,3-*GGTA1* knock-out pigs were produced by somatic cell nuclear transfer (DAI et al., 2002; LAI et al., 2002) and shortly after, the production of homozygous *GGTA1* knock-out (GTKO) pigs was reported in

2003 (PHELPS et al., 2003). The first promising results using these pigs as donors were published in 2005, with maximum graft survival of 179 days (median of 78 days) (KUWAKI et al., 2005). However, xenotransplantation experiments carried out by other groups showed varying survival times (AZIMZADEH et al., 2015), probably related to differences in the immunosuppression regimens (AZIMZADEH et al., 2015), the health status of the recipient as well as of the donor (MOHIUDDIN et al., 2012; HIGGINBOTHAM et al., 2015), or the infection status of certain pathogens, e.g. porcine cytomegalovirus (YAMADA et al., 2014).

Another antibody mediated process, which had to be overcome is acute humoral xenograft rejection (AHXR) (reviewed in KLYMIUK et al., 2010). AHXR is also known as “acute vascular rejection” or “delayed xenograft rejection”, but AHXR reflects most closely the presumed pathogenesis of an antibody-mediated rejection, with likely involvement of complement (reviewed in SCHUURMAN et al., 2003). It appears that AHXR cannot be completely avoided, even if animals receive continuous treatment and even if the donor organ is from an animal transgenic for a human complement regulatory protein (reviewed in SCHUURMAN et al., 2003). The multifactorial aspects of AHXR like endothelial cell activation and injury, destroying the anticoagulant features of the endothelium and so leading to thrombotic microangiopathy and disseminated intravascular coagulopathy are also reflected in histopathologic features. The best documented case is the description of AHXR in a pig-to-nonhuman primate kidney xenotransplantation model, where the histology fully resembled glomerular thrombotic microangiopathy (SHIMIZU et al., 2000). Several strategies to improve graft survival were discussed, like transgenic pigs for human ecto-ADPase (CD39), human thrombomodulin (THBD), endothelial protein C receptor (EPCR), heme oxygenase 1 and tissue factor pathway inhibitor (TFPI) (reviewed in D'APICE & COWAN, 2009). But the best success was achieved by pigs expressing human *THBD* gene (hTBM) under the control of the porcine *THBD* promoter, to overcome the impaired activation of protein C on the porcine endothelium (WUENSCH et al., 2014). Such pigs were generated in 2014 at our institute, on the background of a *GGTA1* knock-out (PHELPS et al., 2003) and human CD46 transgenic (LOVELAND et al., 2004) (hCD46) pig, according to our established work flows (KUROME et al., 2006; KLYMIUK et al., 2012b; RICHTER et al., 2012; KUROME et al., 2013; KUROME et al., 2015) (reviewed in AIGNER et al., 2010b; reviewed in AIGNER et al., 2010a; reviewed in KLYMIUK et al., 2010). *In vitro* studies from different groups show beneficial effects of cells from pigs expressing hTBM (WUENSCH et al., 2014; BONGONI et al., 2016; BONGONI et al., 2017).

The beneficial effect of hTBM transgene expression can also be seen in few heterotopic cardiac xenotransplantation (hCXTx) studies, using hTBM transgenic pigs (ABICHT et al., 2015). Here, donor hearts transgenic for hTBM showed undoubtedly the best graft survival, with minimal thrombocytopenia and bleeding, compared to anti-CD154 treated recipients (MOHIUDDIN et al., 2014; MOHIUDDIN et al., 2016). Additionally to the findings, a very recent study compared the survival of grafts from pigs transgenic for GTKO.hCD46.hTBM (MOHIUDDIN et al., 2016) to those only transgenic for GTKO.hCD46 (SINGH et al., 2019), with the conclusion that all grafts show reduced survival in the absence of THBD transgene expression.

As the median survival time in hCXTx is now 298 days long (minimum 159 days to maximum 945 days) (MOHIUDDIN et al., 2016), it is more than 2-fold longer than recommended by the ISHLT committee on Xenotransplantation for the duration of life-supporting preclinical studies (COOPER et al., 2000). The next logical step was to bring orthotopic cardiac xenotransplantation (oCXTx) to the same level of survival time. But this procedure is far more complex and critical to perform, so there have been relatively few live supporting oCXTs studies (SCHMOECKEL et al., 1998; WATERWORTH et al., 1998; XU et al., 1998; VIAL et al., 2000; BRANDL et al., 2005; BRANDL et al., 2007; MCGREGOR et al., 2008; MCGREGOR et al., 2009). These studies used GTKO.hCD46 transgenic pigs or pigs only transgenic for human complement regulatory proteins, with and without αGal-oligosaccharides to block anti-Gal antibodies.

Xenograft survival in oCXTx studies ranged from 1 to 57 days and in most cases the recipient died because of postoperative complications rather than graft rejection. Although the grafts showed limited histological signs of rejection, gene expression analysis revealed that the hearts were exposed to ongoing immune challenge and endothelial cell activation (BYRNE et al., 2011). These earlier studies, which did not employ the latest immunosuppression regimens from the hCXTx studies indicated, that oCXTx is not limited by cardiac function, but by challenges of immune rejection and postoperative management (MOHIUDDIN et al., 2015).

2.2 Safety aspects

2.2.1 Exogenous pathogens

As post-transplantation infections are commonly seen as side effects in allotransplantations, they might occur in any immunocompromised transplant recipient. The risk of infections is based on the interaction of the immune system of the recipient and the virulence, dose and intensity of specific organisms that are present in the donor graft (reviewed in FISHMAN, 2018).

In allotransplantation, the prevention of donor-derived infections is achieved by donor screening and selection (NELLORE & FISHMAN, 2018). There are multiple existing guidelines for screening, but the time within which organs may be used is limited and so is microbiological screening from either serologies or nucleic acid tests. Furthermore, the data from samples taken for culture are only available after transplantation and therefore only an advice for choosing the fitting antimicrobial treatment of the recipients. Screening criteria may be adapted, respecting the geographic region, travelling history of donor and recipient, local microbiologic epidemiology or individual donor exposures, for example Chikungunya virus (DALLA GASPERINA et al., 2015; PIERELLI et al., 2018), West Nile virus (RAZONABLE, 2016; VELATI et al., 2017) or the severe acute respiratory syndrome (SARS). Sudden clusters of infections among recipients sharing a common donor, or when recipients develop a disease for which they had no exposure, may lead to the conclusion of donor-derived infections.

Recent cases of dengue virus (GUPTA et al., 2016), hepatitis C virus (HCV) (ANONYMUS, 2011), and human immunodeficiency virus (HIV) (ANONYMUS, 2011) have shown, not only deceased organ donors pose a risk for such infections, but also living organ donors. Regardless of all the limitations, unexpected donor-derived infections are estimated to occur in 0,2% of solid organ transplant recipients (WOLFE et al., 2019) (reviewed in ISON & NALESNIK, 2011).

With clinical xenotransplantation studies being more and more within the grasp of researchers, the demand for safe and nonhazardous donor pigs has, thus, become a widely discussed topic. These zoonotic infections, in the xenotransplantation context called “xenozoonosis” or “xenosis” to underline the unique epidemiology, comprise known as well as unknown pathogens. Therefore, the goal of pig husbandry for xenotransplantation is to exclude potential pathogens and to obviate the introduction of any new safety risks, as for

many pig pathogens the potential to cross the species barrier to humans in an immunocompromised patient is not known and for many pathogens microbiological assays have not yet been developed.

But once validated, these assays can be run on a herd or on single donor animals. In contrast to deceased human donor organs, pigs can be screened on a routine basis and even more intensive. Eventually, screening schemes might be created to exclude organisms of risk to human transplant recipients, which allows the selection of swine, free of selected potential pathogens, termed “designated pathogen free” (DPF) (FISHMAN, 1997, 1998, 2001) (reviewed in FISHMAN & PATIENCE, 2004). Some pig pathogens have known zoonotic potential and are known to infect both, human and swine, like hepatitis E virus and influenza virus, many bacteria, like *Salmonella* species, *Pasteurella* species, *Pseudomonas* species, *Yersinia* species, *Campylobacter* species and *Listeria monocytogenes* and fungi, like *Aspergillus* species and *Candida* species. Bacterial and viral infections predominate the scientific literature in the risk analysis, though parasites are increasingly being recognized for their potential to influence on the outcome of a transplantation (FABIANI et al., 2018; LA HOZ & MORRIS, 2019).

Toxoplasmosis, for example may be the most prevalent infection in human, with an estimated 30-50% of the world’s population previously exposed (FLEGR et al., 2014). Toxoplasmosis is caused by a protozoan, called *Toxoplasma gondii* and infection can be foodborne, zoonotic, congenital, from blood transfusion or organ transplants from infected donors (Center for Disease Control, CDC). Screening of all organ donors, not only for allo- but also for xenotransplantation, and recipients is recommended. The most frequent transmission occurs in seronegative recipients from a heart of a *Toxoplasma* IgG-positive donor, unless they receive prophylaxis (LUFT et al., 1983; WREGHITT et al., 1989).

Chagas, an infection with the protozoan parasite *Trypanosoma cruzi*, which is transmitted to humans by reduviid bugs of the subfamily *Triatominae*, causes one of the world’s most neglected tropical diseases, as listed by the World Health Organization (WHO) (COMMITTEE, 2002; HOTEZ et al., 2007; LA HOZ & MORRIS, 2019). After feeding on the host, these blood-sucking insects release infectious trypomastigotes in their feces, which enter the host through the wound, conjunctiva or adjacent mucosa. But infection can also occur through vertical transmission, oral ingestion of contaminated food or water, as well as blood transfusion or organ transplantation (reviewed in BERN et al., 2007; RASSI et al., 2010). Considerations on

screening recipients and donors, should be taken, because of the overall epidemiologic shifts of diseases, as well as more and more people travelling to foreign countries and with more immigrants of foreign countries becoming possible organ donors in developed countries, thus bringing unknown diseases and pathogens with them.

Babesia, another species of protozoan parasites, are transmitted through tick vectors. They are common in most domestic animals (overviewed in UILENBERG, 2006), as well as in humans. Healthy people may clear the infection without treatment, but the resolution of the infection depends on the innate and adaptive immune system. Therefore, infection with Babesia is more severe in persons under immunosuppression, asplenic patients or those infected with certain species (VANNIER & KRAUSE, 2012).

Porcine cytomegalovirus (PCMV), a β- herpesvirus related to the human cytomegalovirus is acquired by piglets very early in life and leads to a lifelong seroconversion and latent viral infection (reviewed in MUELLER & FISHMAN, 2004). PCMV causes systemic disease and eventually leads to transplant failure of xenografts in preclinical studies (YAMADA et al., 2014), like human cytomegalovirus in allotransplantation. Whether PCMV can infect human cells is adversely discussed, with two studies showing on the one hand, possible *in vitro* infection (WHITTEKER et al., 2008) and the other study showing no evidence for this (TUCKER et al., 1999). Available antiviral therapy, for example cidofovir and foscarnet may have therapeutic effect on PCMV viral load in achievable concentrations, but in these concentrations these agents often carry significant toxicity for the transplant recipients (MUELLER et al., 2003). Ganciclovir failed to prevent PCMV infection in various pig-to-baboon solid organ xenotransplantation models. The lack of therapeutic agents makes the establishment of PCMV free swine herd of utmost importance for xenotransplantation.

Hepatitis E virus (HEV) is the main course of acute viral hepatitis worldwide (reviewed in CLEMENTE-CASARES et al., 2016). It is a pathogen of both, humans and swine and often associated with contaminated food or water. It is estimated that one-third of the world population has been exposed to the agent (REIN et al., 2012). Pigs, wild boars and deer are the reservoirs of HEV genotype 3 and 4 (reviewed in KHUROO & KHUROO, 2016). Most human infections occur through intake of undercooked or uncooked meat of infected species, like domestic pigs, especially pig liver and liver products. But also human to human transmission

is possible, through infected blood transfusions and blood component transfusions. Donor screening for HEV in allotransplantation is under serious consideration.

Considering all the pathogens described here and all the potentially hazardous but yet unknown pathogens, the basis of producing donors for xenotransplantation will always be good veterinary practice, good laboratory practice and good manufacturing practice. Pigs might be bred under special conditions, in so-called biosecure environments, with hygiene sluices adapted to the level of biosecurity. The employees and caretakers have to be trained to follow certain steps when entering the housing, maybe showering with full cloth change afterwards, going through air sluices even in the housing, from the highest hygiene level to the lowest. Some authors suggest the use of routine vaccines (GAZDA et al., 2016) and a more or less wide screening program to achieve microbiological safety for clinical trials (GARKAVENKO et al., 2004a; GARKAVENKO et al., 2008a; WYNYARD et al., 2014; SPIZZO et al., 2016; FISHMAN, 2018).

Following, **Table 1** shows a summary of important publications regarding screening concepts for pig herds bred for xenotransplantation.

	Fishman 2018	Spizzio 2016 (IXA)	Wynyard 2014 New Zealand	Garkavenko 2004a+2008a
Bacteria				
Leptospira Serovar Tarrasovi	✓	✓	✓	
Leptospira Serovar Hardjo	✓	✓	✓	
Leptospira Serovar Pomona	✓	✓	✓	
Mycoplasma hyopneumoniae	✓	✓	✓	
Campylobacter	✓	✓	✓	
Yersinia	✓	✓	✓	
E.coli K88		✓	✓	
Salmonella spp.	✓	✓	✓	
Mycobacterium tuberculosis	✓			
Shigella	✓			
nontuberculous mycobacteria	✓			

+ *M.bovis*				
Listeria monocytogenes	✓			
Brucella suis	✓			
Viruses				
MRV			✓	
HERV-K			✓	
PCV1	✓	✓	✓	
PCV2	✓	✓	✓	✓
PLHV	✓			✓
PLHV2		✓	✓	
PCMV	✓	✓	✓	✓
Rotavirus A-C		✓	✓	
Reovirus		✓	✓	
PTV		✓	✓	
PEVB		✓	✓	
PHEV		✓	✓	
HEV	✓	✓	✓	
BVD		✓	✓	
SuHV-1 (AujD)	✓	✓	✓	
PPV	✓	✓	✓	
PRRSV	✓	✓	✓	
EMCV	✓	✓	✓	
PERV		✓	✓	✓
Adenovirus	✓			
Rabies virus	✓			
Influenza virus (human)	✓			
Influenza virus (swine)	✓			
Protozoa/ Parasites				
Toxoplasma gondii	✓	✓	✓	
Ascaris suum	✓			
Cryptosporidium/ Microsporidium spp.	✓	✓	✓	
Echinococcus spp.	✓			
Giardia spp.	✓			
Isospora sp.	✓	✓	✓	
Strongyloides sp.	✓			
Trichinella spiralis	✓			

Trypanosoma spp.	✓			
Fungi				
Aspergillus sp.	✓			
Candida sp.	✓			
Cryptococcus neoformans	✓			
Histoplasma capsulatum	✓			

Table 1 Pathogens that should be excluded from a designated pathogen free pig herd for xenotransplantation, adapted from references (GARKAVENKO et al., 2004a; GARKAVENKO et al., 2008a; WYNYARD et al., 2014; SPIZZO et al., 2016; FISHMAN, 2018).

(MRV: mammalian orthoreovirus, HERV-K: Human endogenous retrovirus K, PCV1 /2: porcine circovirus 1/2, PLHV: porcine lymphotropic herpesvirus, PLHV2: porcine lymphotropic herpesvirus 2, PCMV: porcine cytomegalovirus, PTV: porcine teschovirus, PEVB: porcine enterovirus, PHEV: porcine hemagglutinating encephalomyelitis virus, HEV: hepatitis E virus, BVD: bovine virus diarrhea, SuHV-1 /AujD: suid alphaherpesvirus 1/ Aujezsky's disease, PPV: porcine parvovirus, PRRSV: porcine reproductive and respiratory syndrome virus, EMCV: encephalomyocarditis virus, PERV: porcine endogenous retrovirus)

2.2.2 Endogenous pathogens

Discussing safety aspects, not only the exogenous pathogens are to consider, but also the endogenous pathogens, above all, the porcine endogenous retroviruses (PERVs). Considering that the human acquired immune deficiency syndrome (AIDS) viruses were zoonotic transmission of primate lentiviruses, the transmission characteristics of PERVs have to be studied very carefully (SHARP PAUL et al., 1995; GAO et al., 1999).

According to the current classification of the International Committee on Taxonomy of Viruses (ICTV) PERVs belong the Retroviridae family, subfamily of Orthoretrovirinae, the genus Gammaretrovirus and the species Porcine type – C oncovirus (ICTV, 2019).

Resembling virus-like particles as those seen in baby hamster kidney cell line (BHK-21) and murine cells infected with murine leukemia virus (MLV) PERVs were first described in 1970 (BREESE, 1970). They are close related to MLV, feline leukemia virus (FeLV), gibbon ape leukemia virus (GaLV) and koala retrovirus (KoRV) (reviewed in DENNER, 2007; reviewed in DENNER, 2008a). Sequences similar to mouse endogenous retroviruses indicate that PERVs originated from mouse endogenous retroviruses about 7.4-8.3 million years ago, which correlates with the point of separation between pigs and peccaries (TONJES & NIEBERT, 2003; NIEBERT & TONJES, 2005; TANG et al., 2016). Recent scientific findings reveal, that retroviruses themselves are much more older, have ancient marine roots and originated over 450 million years ago in the early Palaeozoic Era (AIEWSAKUN & KATZOURAKIS, 2017).

PERVs are characterized by the possession of the enzyme reverse transcriptase (RT) (reviewed in LOPATA et al., 2018). RT transcribes genomic ribonucleic acid (RNA) into double stranded deoxyribonucleic acid (DNA). This double stranded DNA, which is then called the provirus, integrates itself unperceived into the host's genome. This also affects the germ line of the host, which gives the provirus the possibility to be passed from one generation to another, thus becoming an endogenous retrovirus (ERV) (HAYWARD & KATZOURAKIS, 2015; HAYWARD, 2017) (reviewed in WEISS, 2006). The once into the germline integrated provirus is inherited as retroviral insertion to the host's descendants following the Mendelian rules, which characterizes ERVs.

This presents us with the challenge that PERVs cannot be eliminated by the standard hygiene measurements and methods, like cleaning, disinfection or air filtration but other methods

than currently employed in barrier facilities to exclude exogenous pathogens (SCHUURMAN, 2009) (reviewed in SCOBIE & TAKEUCHI, 2009).

With porcine kidney cell lines spontaneously producing C-type retrovirus particles (ARMSTRONG et al., 1971), the question if they can infect human cells arose (PATIENCE et al., 1997). Followingly, the three replication-competent subfamilies PERV-A, PERV-B and PERV-C were identified (TAKEUCHI et al., 1998). The two subfamilies PERV-A and PERV-B were found of being capable to infect human cells *in vitro* (LE TISSIER et al., 1997). These two human-tropic PERVs can be found in all pigs (DENNER & SCOBIE, 2019). PERV-C on the other hand, is not ubiquitous in the pig population and can only infect pig cells.

As it is not yet sure, if PERVs can infect human cells *in vivo*, they have to be seen as a thread to xenotransplant recipients, especially, as mentioned above, that it was shown, that PERVs can infect human cells *in vitro* (LE TISSIER et al., 1997; PATIENCE et al., 1997; TAKEUCHI et al., 1998; SCOBIE & TAKEUCHI, 2009). But still it has to be kept in mind, that the envelop (*env*) gene determines the viral tropism, consequently the virus receptor (TAKEUCHI et al., 1998; LEE et al., 2006) and so far, only the receptor for PERV-A has been identified (reviewed in LOPATA et al., 2018). In pigs it is called porcine PERV-A receptor (PoPAR), in baboons, baboon PERV-A receptor 2 (BaPAR-2) and in humans are two known receptors, human PERV-A receptor 1 (HuPAR-1) and human PERV-A receptor 2 (HuPAR-2) (ERICSSON et al., 2003). HuPAR-1 expression is more widespread (YONEZAWA et al., 2008; MARCUCCI et al., 2009; YAO et al., 2010), but it is peculiarly enhanced in the brain and the salivary glands (NAKAYA et al., 2011). Contradictory, the expression of HuPAR-2 is elevated in the placenta and the small intestine. Although expression of these receptors is nearly ubiquitous in most human tissues examined in the study by Ericsson et al., other *in vitro* studies have shown, that only few human and nonhuman primate cells were permissive for productive PERV-A infection, even if they were susceptible for PERV-A entry (WILSON et al., 2000; RITZHAUPT et al., 2002). It was shown that HuPAR-2 is on average 11-fold more functional than HuPAR-1 regarding PERV-A infection and this increase in infectivity was no matter of any difference in viral envelope binding, but in fact is due to the inherent biological variability of viral infection testing strategies (MARCUCCI et al., 2009). PERV transmission has been confirmed to human peripheral blood mononuclear cells (PBMCs) (CLEMENCEAU et al., 2001; SPECKE et al., 2001), primary endothelial cells and primary aortic smooth muscle cells (SPECKE et al., 2001), vascular fibroblasts and mesangial cells (MARTIN et al., 2000), human embryonic kidney (HEK)

293 cells (MARTIN et al., 1998; LEE et al., 2008) and normal dermal human fibroblasts (NHDFs) (KIMSA et al., 2013). Nonetheless, *in vivo*, PERV transmission has never been observed among patients after pancreatic islets xenografts (HENEINE et al., 1998; GARKAVENKO et al., 2004b; VALDES-GONZALEZ et al., 2010; MOROZOV et al., 2017), patients upon receipt of fetal porcine neuronal cells (FINK et al., 2000), recipients of porcine liver cell-based bioartificial liver (DI NICUOLO et al., 2005; DI NICUOLO et al., 2010), porcine skin graft recipients (SCOBIE et al., 2013) and lastly butchers, who are on a daily basis in close contact with pig tissue (PARADIS et al., 1999; DENNER, 2008b; DENNER & TONJES, 2012). Therefore, it still is questionable if the human cells used in *in vitro* studies, for example the widely used HEK293 cells are representative, because of different virus receptors and different levels of expression in different tissues, which too, are very likely to be influenced by various factors, like dependency networks, that cannot be mimicked in cell culture in exact the same way as it is in a living organism.

Recently, studies with inbred miniature swine and melanoma-bearing pigs described a human-tropic, replication competent, recombinant, high-titer PERV-A/C, which de novo integrated into the genome of the spleens of the pigs, but was not found in the germ line (BARTOSCH et al., 2004; WOOD et al., 2004; MARTIN et al., 2006; DIECKHOFF et al., 2007; DENNER, 2008c; KARLAS et al., 2010). PERV-A/C was transmitted to human cells *in vitro*, with the receptor binding domain of PERV-A combined with PERV-C related sequences (OLDMIXON et al., 2002; BARTOSCH et al., 2004). Most of the studies used PBMCs derived from miniature pigs, to demonstrate the transmission of recombinant PERV-A/C. These experiments have been repeated with PBMCs from the Auckland Islands pigs of New Zealand with the result, that no PERV was transmitted, either to human or to pig cells (GARKAVENKO et al., 2008c; GARKAVENKO et al., 2008b). In a preclinical pig-to-primate islet cell xenotransplantation study drawn out in 2008, where also cells from the Auckland Islands pigs were used, there was no evidence of virus transmission to the nonhuman primates (GARKAVENKO et al., 2008a). To simulate the situation after a possible xenotransplantation to humans *in vitro*, serial cell-free passages were performed on human cells, which resulted in the increase of the titer of the virus (WILSON et al., 2000; DENNER et al., 2003). This increase was associated with genetic changes in the viral long terminal repeats (LTR), which was similar when PERV-A was passaged (WILSON et al., 2000; SCHEEF et al., 2001). But when compared with the paternal PERV-A, mutations in the *env* gene were identified, that also might be responsible for high titers

(HARRISON et al., 2004). Furthermore, shows PERV-A/C an enhanced RT activity compared to PERV-A (WOOD et al., 2009). To avoid the assembly of recombinant, high-titer PERV-A/C it is strongly recommended to avoid pig strains carrying ecotropic PERV-C for breeding animals for xenotransplantation (DENNER et al., 2009).

Several efforts to minimizes the risk of PERV transmission have been made, but all only had limited success. To increase viral safety by RNA interference, transgenic pigs expressing a PERV-specific small hairpin RNA were generated, in which expression of PERVs was reduced (DIECKHOFF et al., 2008; RAMSOONDAR et al., 2009; SEMAAN et al., 2012). Another attempt was the design of vaccines (FIEBIG et al., 2003; KAULITZ et al., 2011) or the use of antiretroviral drugs (such as azidothymidine) (QARI et al., 2001; STEPHAN et al., 2001; SHI et al., 2007) (reviewed in DENNER, 2017) and PERV elimination by using zinc finger nucleases (SEMAAN et al., 2015) (ZFNs) and transcription activator-like effector nucleases (DUNN et al., 2015) (TALENs).

A successful inactivation of all 62 copies of the PERV *pol* gene in the PK15 cell line (YANG et al., 2015) was achieved by usage of the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system (JINEK et al., 2012; CONG et al., 2013; MALI et al., 2013). With this approach it was possible to effect a 1000-fold reduction of infectivity of PERV of human cells (YANG et al., 2015), which demonstrates, that PERVs can be inactivated in pig genomes for clinical application of pig-to-human xenotransplantation. With only little adaption, this technique was used to produce 37 PERV-inactivated piglets by somatic cell nuclear transfer (SCNT), from which 15 piglets remained alive to conduct long term studies to monitor the impact of PERV-inactivation and gene editing on animals (NIU et al., 2017).

2.3 Requirements claimed by regulatory authorities

2.3.1 World Health Organization (WHO)

In the documentation center on the WHO website, there are several guides, recommendations and regulations available, dealing with xenotransplantation and different topics regarding xenotransplantation.

The "Guide on Infectious Disease Prevention and Management" from 1998 deals with the requirements for xenotransplantation (WHO, 1998). It discusses the possible dangers of introducing animal-origin infectious agents into human population and how to best avoid these and related dangers. It guides the reader through the process of developing a xenotransplantation infectious agent exclusion list and designing a surveillance program.

First, the risk of exposure, the potential for introducing the infectious agent into the recipient, must be recognized and assessed. If a risk of exposure is given, the potential for establishment in the new host must be then considered and evaluated: Does the establishment only require direct contact or is adaption or genetic alteration required? Is the establishment restricted to the transplanted tissue or is there a possibility to disseminate throughout the new host? If this could be the case, the likelihood of disease production in the general population must be assessed (WHO, 1998).

In Annex I a list is attached with suggested criteria for consideration when developing a xenotransplantation infectious-agent exclusion list. But any list must be drafted with professional judgement and cautious flexibility, to assure the list reflects the best possible integration of technical feasibility and risk acceptability. These two factors will, to a large extent, dictate the number and type of agents in the xenotransplantation context (WHO, 1998). Further, should the list be generated by a consortium of experts representing all scientific fields involved in xenotransplantation. Periodic reviews and updates of the list should be out of question. Obviously, the generation of the specific agent exclusion list will be or is, an enormous but necessary task (WHO, 1998).

The "Guide on Infectious Disease Prevention and Management" also discusses how to minimize the risk to public health, which includes, among others, the establishment and implementation of stringent selection requirements for prospective tissue and organ donor-

animals (WHO, 1998). This implies the maintenance, licensing of xeno-dedicated animal colonies, which are closely monitored in a surveillance program.

To give the best possible infectious disease prevention, in the case of xenotransplantation, the surveillance program should not be restricted to be donor animals, but also a practical and clinically feasible recipient follow-up must be designed, to detect and contain unrecognized or emerging infectious agents (WHO, 1998).

The "OECD/WHO Consultation on Xenotransplantation Surveillance: Summary Report" from 2001 (WHO, 2001b) summarizes the topics, issues and considerations discussed at the OECD/WHO Consultation on Xenotransplantation Surveillance held in Paris on 4-6 October 2000. The purpose was to bring together epidemiologists, infectious disease specialists, clinicians, industry, government and international organization representatives and others working in public health and xenotransplantation research to discuss the following topics:

- "What is a xenogeneic infectious disease event? What are some of the problems associated with the development of standardized case definition?"
- "What can be learned about characteristics of already existing and successful surveillance systems that might be applicable?"
- "What are particular characteristics associated with xenotransplantation that must be accommodated in any developed surveillance systems for xenogeneic disease events?"
- "What ethical considerations will need to be incorporated into a xenogeneic disease event surveillance system?"
- "What might be a practical framework for international surveillance?"

Concluding, it can be said that the broad view of participants was, that an international surveillance system for xeno-associated infectious disease events is needed, regarding the number of clinical trials and the potential risk of xenogeneic pathogens (WHO, 2001b).

The "WHO Guidance on Xenogeneic Infection/Disease Surveillance and Response: A Strategy for International Cooperation and Coordination" (WHO, 2001a) aims at facilitating the considerations for development and implementation of an international xenogeneic infection or disease event surveillance network for efficiently and effectively detecting, reporting and responding to such events using internationally harmonized, cooperative and coordinated surveillance activities (WHO, 2001a).

The resolution WHA57.18 of the 57th World Health Assembly (WHO, 2004) urges member states to carry out xenogeneic transplantation only when effective national regulatory control and surveillance mechanisms are in place, to cooperate in the formulation of recommendations and guidelines and to support international collaboration and coordination for the prevention and surveillance of xenogeneic infections. But it also requests the Director-General, amongst others, to provide technical support in strengthening capacity and expertise in the field of xenogeneic transplantation.

This was followed by first Xenotransplantation Advisory Consultation in Geneva in 2005 (WHO, 2005), succeeded by the "First WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials", whose recommendations were published as the "Changsha Communiqué" in 2008 (WHO, 2009).

The "Second WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials" was held in Geneva, Switzerland in October 2011 (WHO, 2011) and deals, among other topics, with the current status of xenotransplantation science and practice and the discussion and refinement of draft guidance for infectious disease surveillance, prevention and response.

In collaboration between WHO, International Xenotransplantation Association (IXA), and the Third Xiangya Hospital of the Central South University, Changsha, Hunan, China, the 10-year anniversary of the "Changsha Communiqué" was celebrated with the organization of the "Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials" in December 12-14, 2018 (HAWTHORNE et al., 2019). The proposed revisions of the WHO documents resulted in the formulation of the draft "Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials, The 2018 Changsha Communiqué.", which was submitted to WHO in February 2019 for WHO and World Health Assembly consideration. If it obtains approval, the 2018 Changsha Communiqué will be posted on the websites of WHO, IXA, and The Transplantation Society (TTS), and published in Xenotransplantation (HAWTHORNE et al., 2019).

2.3.2 Food and Drug Administration (FDA)

The U.S. Food and Drug Administration (FDA) currently has three guidance documents regarding xenotransplantation published.

The “Guidance For Industry: Public Health Issues Posed by the Use of Nonhuman Primate Xenografts in Humans” provides guidance to industry and researchers concerning the use of nonhuman primates as the source of cells, tissues and organs, the potential public health risks posed by nonhuman primate xenografts, the need for further scientific research and evaluation of these risks, particularly infectious agents and the need for public discussion concerning these issues (FDA, 1999).

This guidance was followed by the U.S. Public Health Service (PHS) “PHS Guideline On Infectious Disease Issues in Xenotransplantation” in 2001 (FDA, 2001), which was developed to identify general principles of prevention and control of xenogeneic infectious diseases that may pose a risk to public health. It addresses the public health issues related to xenotransplantation and recommends procedures to minimize the risk of transmission of infectious agents to the recipients, medicinal personnel, close contacts and the general public.

The recommendations given in the “Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans” by the Food and Drug Administration (FDA), from 2003 and updated in 2016 claim, among others, that you should only derive animals from closed herds with documented health screening programs, that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (FDA, 2003). Furthermore, should the facilities not be built near any other agricultural or manufacturing facilities, as they are a source of infection. You should provide standard operation procedures (SOPs) for any activity that has in the broadest sense to be done with the animals or the animal facility. For example, to define the DPF status of the donor animals and the facility, initial screening and routine monitoring have to be done and therefore protocols and SOPs of these monitoring schemes should exist. In order to establish a list of pathogens to be screened for and which diagnostic test is appropriate, the FDA suggest to consult experts, such as infectious disease consultants, virologists, microbiologists, accredited microbiological laboratories, and veterinarians (FDA, 2003).

Also, the storage and delivery of feed, water and any other consumables should be described, as well as it is advised to keep recordkeeping of the manufacturer, batch numbers in order to allow backtracking (FDA, 2003).

SOPs for caretakers should include entry and exit procedures, clothing requirements and all other interactions that may take place between them and the animals. A documented training program for the caretakers and personnel according to current good manufacturing practices should regularly take place (FDA, 2003).

Those are just few examples of the recommendations given in the "Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans" by the FDA, which guides through the whole product manufacturing process, starting with the source animal and also advising on how to store the samples.

2.3.3 European Medicines Agency (EMA)

The "Committee For Medicinal Products For Human Use (CHMP)" from the European Medicines Agency (EMA) issued the "Guideline On Xenogeneic Cell-Based Medicinal Products" in 2009 (EMA, January 1, 2010). The guideline should be read together with the introduction and general principles (4) and part 4 of the Annex I to Directive 2001/83/EC, the Regulation (EC) No 1394/2007 on Advanced Therapy Medicinal Products and the Directive 2001/18/EC, when cells are obtained from genetically modified animals. First, the authors define xenogeneic cell-based therapy as the use of viable animal somatic cell preparations, which are suitably adapted for either implantation/infusion into a human recipient or extracorporeal treatment by bringing animal cells into contact with human body fluids, tissue or organs, where the principal objective is reconstitution of cell, tissue or organ functions (EMA, January 1, 2010). This guideline is an annex to the guideline EMEA/CHMP/41086/2006 and deals specifically with scientific requirements unique for xenogeneic cell-based medicinal products. The main issues of the "Guideline On Xenogeneic Cell-Based Medicinal Products" are the source and the testing of the animals, manufacture and quality control and non-clinical and clinical development of xenogeneic cell-based medicinal products. Furthermore, it deals with public health aspects to ensure proper surveillance for infections, especially zoonoses. Sources for xenogeneic material can be non-transgenic, transgenic and genetically-modified animals.

Regarding quality and manufacturing aspects, there are three critical points given in the guideline: the source animals, the procurement and the processing of the organs, tissues and cells (EMA, January 1, 2010). The manufacturing facility should be good manufacturing practice (GMP) approved and separated from the animal facility. The health status of the animals should be monitored and documented, with special attention to organ and tissue specific pathogens. Further should the origin of the animals be fully described, e.g. typically for consumption or for laboratory use and they should be at least be specific pathogen free (SPF) and held under SPF conditions. The cells, tissues and organs for manufacturing xenogeneic cell-based medicinal products should only be produced from animals bred in captivity, in a barrier facility, and only bred for this special purpose. Under no circumstances should cells, tissues and organs from wild animals or from abattoirs be used. Additionally, the tissue of founder animals should not be used.

Cells, tissues or organs may be obtained from genetically modified animals, or may be obtained by ex vivo genetic modification. In any case, genetically modified animals must be fully characterized and have to comply with applicable European legislation. Animal cells from genetically modified animals used as active substance should comply with “Note for Guidance on the Quality, Preclinical and Clinical aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99) (EMA, January 1, 2010). The guidance on risk assessment of gene therapy medicinal products in the guideline EMEA/CHMP/GTWP/125491/2006 can be useful for xenogeneic cell-based medicinal products as well.

SOPs for the following procedures should be installed to avoid incidents that negatively affect the health of the herd or colony and thus could negatively impact on the barrier facility or the SPF status of the herd (EMA, January 1, 2010) : detailing the housing of the animals and the containment conditions. Water, bedding, source and handling of feed, including feeding. Entry and exit of the animals, animal transportation, identifying individual animals and recording their movements to, through and out of the facility. Disposition of animal tissues and dead animals and removal from production and disposal of the animals and their by-products. Performance and monitoring of health screening and isolation and quarantine.

The “Guideline On Xenogeneic Cell-Based Medicinal Products” advises to use protocols for monitoring the herd and to introduce a herd health surveillance system with a complete documentation of all veterinary care that the animals received. The specific screening routines

should include physical examination and laboratory tests, where all infectious agents known to potentially infect the source species have to be considered. It recommends that there is no use of any antibiotics or vaccines in the source animals. But, if treatment of animals with any medicines is necessary for animal welfare reasons, the impact on the product should be evaluated and discussed with the competent authority. Any use of vaccines must be justified (EMA, January 1, 2010).

As mentioned above the testing programs for source animals should be tailored for the purpose of the product and updated periodically to reflect advances in the knowledge of infectious disease. Adequate and validated diagnostic assays and methods have to be available before initiating clinical trials. Some pathogens to be considered are given in the "Guideline On Xenogeneic Cell-Based Medicinal Products", e.g. endogenous retroviruses (ERV e.g. porcine ERV), infectious agents of humans relating to receptors expressed by transgenic animals (CD46 (membrane cofactor protein, MCP-1) as the cell-surface receptor for measles virus), antibiotic-resistant bacteria, geographically important infectious agents such as *Trypanosoma cruzi* or African Swine Fever.

Adequate archiving is another crucial point to be discussed, because long-term archiving of tissue samples, cell preparations and paper records will be necessary (EMA, January 1, 2010). Records should be kept for 30 years, which makes an established and validated archiving plan inevitable, to ensure traceability and the possibility for look-back. All records concerning the herd, e.g. feeding and health records, source animal health documentation, should be archived for a period at least equal to that of the archived tissue samples.

Giving a very good overview of the regulatory landscape, especially of cell therapy products in Europe, for example pancreatic islets and hepatocytes, the Review "Regulatory aspects of clinical xenotransplantation" from Henk-Jan Schuurman (reviewed in SCHUURMAN, 2015) has to be mentioned at this place.

2.3.4 Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)

The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International is a private, nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs (AAALAC, 2019). It is proposed that facilities should achieve accreditation by AAALAC (reviewed in SCHUURMAN, 2015). By earning a voluntary AAALAC accreditation, the companies, universities, hospitals, government agencies and other research institutions show their commitment to responsible animal care and use (AAALAC, 2019). This is done through the accreditation process in which research programs demonstrate that they meet the minimum standards required by law, and are even going the extra step to achieve excellence in animal care and use. To acquire accreditation the "Guide for the Care and Use of Laboratory Animals" shall serve as a basic guide to the establishment of specific standards.

In the "Guide for the Care and Use of Laboratory Animals" one of the key-concepts is, that all the people working, using, producing or caring for animals for testing, research or teaching must assume that they are responsible for the well-being of these animals (NRC, 2011). It establishes the minimum ethical, practice and care standards for researchers and their institutions.

In chapter one, laboratory animals are defined as any vertebrate animal, e.g. traditional laboratory animals, agricultural animals, wildlife and aquatic species, produced for or used in research, testing or teaching (NRC, 2011). All personnel involved with the care and use of animals must be adequately educated, trained and/ or qualified in the basic principle of laboratory animal science to help ensure high-quality science and animal well-being. The opportunity and the support for regular professional development, training and continuing education should be given by the institutions (COLBY et al., 2007).

Further, in chapter two with the heading "Animal Care and Use Program", it is stated, that the selection of appropriate housing systems for the experimental animals should be carried out by specialists, as it needs professional knowledge and depends on the expected hazards and experiments that will be carried out. When experiments involving hazards are performed, special attention should be given to procedures for animal care and housing, storage and distribution of the agents, dose preparation and administration, body fluid and tissue

handling, waste and carcass disposal, items that might be used temporarily and removed from the site and of course personnel protection (NRC, 2011).

Chapter three deals with environment, housing and management. Here the aspects like temperature, humidity, ventilation, air quality and illumination are described and the importance of environmental enrichment to enhance the animal well-being is stressed-out. It is further described how to estimate the space needs of animals correctly and what factors need to be considered regarding space estimation. The same issues are discussed for aquatic animals.

Chapter four, “Veterinary Care”, is dedicated to the employment of an adequate veterinary care program, which consists of the assessment of animal well-being and the effective management of: animal procurement and transportation, preventive medicine, clinical disease, disability or related health issues, protocol-associated disease, surgery and perioperative care, anesthesia and analgesia and euthanasia (NRC, 2011).

The last chapter deals with the physical plant itself and gives advice on how to plan, design, construct and properly maintain an animal experimental facility. It discusses the advantages and disadvantages of a centralized animal facility versus a decentralized facility and illustrates what has to be considered when special facilities, like a surgery, imaging or a whole body irradiation unit have to be built (NRC, 2011).

As pigs are the preferred donor species in xenotransplantation, another very useful guide for animal housing, especially for agricultural animals is the “Guide for the Care and Use of Agricultural Animals in Research and Teaching” by the Federation of Animal Science Societies (FASS) (FASS, January 2010). In the USA, the “Guide for the Care and Use of Laboratory Animals” by the National Research Council (NRC) (NRC, 2011) and the “Guide for the Care and Use of Agricultural Animals in Research and Teaching” (FASS, January 2010) are the two guidelines which may be utilized when managing programs engaged in research, testing, and teaching with agricultural animals (SWANSON et al., 2018). Also, chapter 23 “Agricultural Animals” from the book “Management of Animal Care and Use Programs in Research, Education, and Testing” (2nd edition) by Janice C. Swanson, Larry T. Chapin, and F. Claire Hankenson provides an overview of the considerations regarding the care and use of agricultural animals, their environment and housing and it highlights the available resources to assist program managers, veterinarians, and research staff (SWANSON et al., 2018).

2.3.5 Federation of European Laboratory Animal Science Associations (FELASA)

The Federation of European Laboratory Animal Science Associations (FELASA) is an association from different European societies working in the field of laboratory animal science (BFR, 2019). FELASA was founded in 1978 and the German "Gesellschaft für Versuchstierkunde/Society of Laboratory Animal Science" (GV-SOLAS) is also a member. FELASA organizes scientific congresses on a regular basis and publishes guidelines and recommendations regarding the whole field of laboratory animal science. GV-SOLAS is a registered society, which deploys itself for the responsible handling of laboratory animals (GV-SOLAS, 2013).

In the report "FELASA recommendations for the health monitoring of breeding colonies and experimental units of cats, dogs and pigs" of the FELASA Working Group on Animal Health (REHBINDER et al., 1998) the authors give detailed information about the importance of an animal health monitoring program and the purpose of this recommendations, namely to harmonize the procedures, achieve similar standards of testing and that reports have a common standard and format within the FELASA member countries (REHBINDER et al., 1998).

Eleven general considerations have to be made according to the recommendations. For example, that the local variations through Europe affect the number of agents that have to be monitored, or, if diseases are declared absent by a national authority, they do not need to be monitored. Depending on local circumstances, e.g. colony size, regional prevalence of specific organisms or existence of national monitoring schemes, actual practice may exceed these recommendations.

These recommendations are intended for all breeding colonies and experimental units of cats, dogs and pigs in biomedical research (REHBINDER et al., 1998). Further should each breeding unit be considered as a self-contained microbiological entity. SOPs must be available in the monitoring laboratories. Furthermore, should they follow GLP principles and participate in a Quality Assurance Program.

If a pathogen is identified or antibodies to it are detected it must be declared as present, with the exception of vaccinated animals. The presence of antibodies against organisms for which it has not been vaccinated is an indicator of infection in the colony. It should be kept in mind, that negative results only state, that the presence of an agent monitored has not been

demonstrated in the animals screened by the tests used. Therefore, the results are not necessarily a reflection of the health status of all animals in the unit (REHBINDER et al., 1998).

The written copies of vaccination and deworming policies should be provided and the brand, date and dose must be recorded when deworming or vaccination is done. Further should the information on the manufacturer, batch number and expiry date of the product be recorded. In non-barrier facilities most cats, dogs and pigs are vaccinated according to general conditions of the breeding colony, buyers' requirements, on request and according to import/export regulations (REHBINDER et al., 1998).

The health inspection of the colony should be assessed by a veterinarian at least every month and all animals must be observed daily by an animal technician (REHBINDER et al., 1998). The samples for the routine health monitoring have to be taken from live animals, however they can be extended by samples obtained from dead or euthanized animals. Bacteriology, serology and parasitology are preferably monitored individually.

At least every three months not less than ten randomly selected animals should be sampled, or sampling should take place according to the respective national disease control programs and import/export regulations. The main purpose of this health monitoring of experimental units is to provide the researchers with data on variables (pathogens, agents, diseases) that might influence their experiments (REHBINDER et al., 1998). These data are part of their work and have to be considered when interpreting the results. Therefore, results of health monitoring programs should be included in scientific publications.

Where breeders or users of laboratory animals are reporting the results of a health monitoring program, which is in full accordance with the recommendations published by FELASA, the report should be titled "FELASA-*Approved Health Monitoring Report*" or they may also use the words "in accordance with FELASA recommendations" (REHBINDER et al., 1998). But this wording can only be used if the methods, frequency, sample size, species-list of organism monitored and reported are in full accordance with the recommendations published by FELASA (REHBINDER et al., 1998).

In the report "FELASA recommendations for the health monitoring of breeding colonies and experimental units of cats, dogs and pigs" are lay-out advises for the "FELASA-Approved Health Monitoring Report" for each species, namely cat, dog and pig given (REHBINDER et al.,

1998). Additionally, there are tables of the viral, bacterial and parasitic agents and suitable test methods for each pathogen shown.

3 Material and Methods

Major elements of my thesis have been included in different publications. For those parts, the relevant Material and Methods have been described in the respective manuscripts. The following section describes the Material and Methods used for tracing PERV-C proviruses in the genomes of the xenotransplantation breeding herds.

3.1 PERV-C detection

PCRs have been run on DNA isolated from porcine tissue by using Nexttec™ 1-Step Tissue & Cells DNA isolation kit (Nexttec™ Biotechnologie GmbH, Leverkusen, Germany) or the DNAeasy® Blood&Tissue kit (Qiagen, Hilden, Germany), according to the manufacturers´ protocols. For each PERV-C integration site, several primer pairs have been designed to detect either the abundance of solo-LTR, the entire provirus or the untouched genomic sites prior to integration. Eventually, one primer pair was optimized for reliable detection of each PCR.

Sequences of the primers are listed in **Table 2**. Eventually, for each PCR a pair of primers have been chosen for routine detection under standard running conditions (**Table 3** and **Table 4**). Each PCR was composed of the same constituents at the same volumes (**Table 5**).

Primers used to establish PCRs to detect LTR and provirus sequences in the pigs, as well as to investigate the sites in the pigs' genomes without PERV-C, where other pigs have PERV-C integrated are listed in **Table 2**. Primers used with the cycler protocol "PERVC1" are marked green and primers used with the cycler protocol "PERVC3" are marked blue.

primer	manufacturer	sequence
chr14:62_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-TGTGGAATGATAGATACTGGTTAAGAG
chr14:62_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-AGACTAGGAGTCAGCAGAGTTTA
chr14:62_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-GACAATTTGCACATAGCAGTGTA
chr14:62_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-GGTGAGCTGAGGAAGGATTT
chr14:62_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-TGATCACTACAGTCTGCCAAAG
chr14:62_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-GTCCAATGGTCGAGAGTCAAA

chr14:62_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-GTACATGCAGCCAACTGGTCA
chr14:62_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-AAGCAGGCAAAAGAGTCGGA
chr4:48_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-AAGTGTCCTTGACTCCAGAAAG
chr4:48_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-GGCCAGTGTCCCATCTTAAA
chr4:48_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-GACTCCAGAAAGCCACAGTT
chr4:48_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-GCCTTGACACAACAAGAGTTTC
chr4:48_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-TCAGGGAATGGTCAATGTATGG
chr4:48_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-ACTGCTTTAATAGCCAGGATAA
chr4:48_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-AGGATGCAGGCTTGAGACAG
chr4:48_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-CCTGAGAGGACAGCTGCAAA
scf200_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-GCTGTCCGTTCTCATCTCAAA
scf200_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-CAAGTAGCAGTTCCACCTTGTA
scf200_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-TCCTGAAGACGGTGTCCTCT
scf200_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-TTAAAACTGCCGAGGGAGCC
scf200_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-CATGGCCTCCTAAGCTTTCT
scf200_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-TGCCAAGGTCCCTTCTTAAC
scf200_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-GTGCATACGCGGTTTCCTTC
scf200_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-GGACAGCTGCAAACCGAAAG
chr11:29_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-TCTTGAACTACACACAGACATCA
chr11:29_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-TCTTGTGACAGAGTATTTCCAGCA
chr11:29_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-TATTTAAACCATATGCCAGATAAGCAC
chr11:29_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-TGCTAAGTATACATAACATTTGACATTCT
chr11:29_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-TCTTGAACTACACACAGACATCATA

chr11:29_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-TGCCAAGGTCCCTTCTTAAC
chr11:29_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-TTCTTGAACTACACACAGACATCA
chr11:29_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-GTTTAACCCATGGCGGAGGA
scf141_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-CTGCATTCTGCAAAGGGAAAC
scf141_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-CTGAGCCAAGCCGCATTA
scf_141_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-CACTGCTGCTTGGCTGGTGGTA
scf_141_LTR_r2	biomers.net GmbH, Ulm, Germany	5'-ACACAAAGCGCGCTTCTAGGA
scf_141_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-CCTATCAGGAGAAAGAGACTT
scf_141_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-TAATGCGGCTTGGCTCAG
scf_141_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-CCAAAGCCCGTCTAGCAGGAAA
scf_141_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-ACACAAAGCGCGCTTCTAGGA
chr7:23_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-GTATTGTTCTGGAGGGCTTGTG
chr7:23_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-GTAAACCATATATTATTC
chr7:23_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-ATCTTCACCACGGCTGTAGCT
chr7:23_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-ATGGAACTTCCCAGGC
chr7:23_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-CACGGCTGTAGCTCAATCTTAT
chr7:23_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-GGTCCCTTCTTAACCTGAACTG
chr7:23_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-TCTGCCTGGTGGGTTGAAAG
chr7:23_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-GGACAGCTGCAAACCGAAAG
chrX:32_LTR_f_1	biomers.net GmbH, Ulm, Germany	5'-GTGTGAGAGTGTGTTCTAGT
chrX:23_LTR_r_1	biomers.net GmbH, Ulm, Germany	5'-GAATCCTTCCCTGGAATAC
chrX:32_LTR_f_2	biomers.net GmbH, Ulm, Germany	5'-AATATTCATGAGGTTGATG
chrX:32_LTR_r_2	biomers.net GmbH, Ulm, Germany	5'-TTCACAAAACTAGAACAATCG

chrX:32_prov_f_1	biomers.net GmbH, Ulm, Germany	5'-GATGGTGTCCTGTCGTA
chrX:32_prov_r_1	biomers.net GmbH, Ulm, Germany	5'-TCGGTCCTCTGACCG
chrX:32_prov_f_2	biomers.net GmbH, Ulm, Germany	5'-TAAGCCCTTGTCAGTTGCA
chrX:32_prov_r_2	biomers.net GmbH, Ulm, Germany	5'-AGATCCAGACACACGTGACC
PervCexc1.3_1f	biomers.net GmbH, Ulm, Germany	5'-CGGAAGTGACGACACAGGAA
PervCexc1.3_1r	biomers.net GmbH, Ulm, Germany	5'-TGAATGTGCACGACGGGTTA
PervCexc1.3_2f	biomers.net GmbH, Ulm, Germany	5'-CTGACAGGTAATGGGTCATCAG
PervCexc1.3_2r	biomers.net GmbH, Ulm, Germany	5'-GACGGGTTCAAGAGGTGAAA
PervCexc1.3_3f	biomers.net GmbH, Ulm, Germany	5'-TTTCCGCATCCGATAGCCTC
PervCexc1.3_3r	biomers.net GmbH, Ulm, Germany	5'-GGGACCCCTGTTTCTACAGC
PervCexc1.3_4f	biomers.net GmbH, Ulm, Germany	5'-AGCCACGCTAATCCGAAACA
PervCexc1.3_4r	biomers.net GmbH, Ulm, Germany	5'-TTTCCTTTCTCCCGCTTCCC
PervCexc1.20_1f	biomers.net GmbH, Ulm, Germany	5'-TTGCCTGCCTTGCTAATCTC
PervCexc1.20_1r	biomers.net GmbH, Ulm, Germany	5'-GCTTCTGGTTGTCCCTTCTATG
PervCexc1.20_2f	biomers.net GmbH, Ulm, Germany	5'-TCGGTTCTCCCTCTTTCTCT
PervCexc1.20_2r	biomers.net GmbH, Ulm, Germany	5'-TTTAAGCAGGGCTGGTAAGG
PervCexc1.20_3f	biomers.net GmbH, Ulm, Germany	5'-CCTTCGCTCTCCCAGGATTC
PervCexc1.20_3r	biomers.net GmbH, Ulm, Germany	5'-ATAAAGCACCCTGGAGGCAC
PervCexc1.20_4f	biomers.net GmbH, Ulm, Germany	5'-CAACTGTCCTGTCCATCCCC
PervCexc1.20_4r	biomers.net GmbH, Ulm, Germany	5'-CCCCTTTTCCTGACATCCCC
PervCexc5.23_1f	biomers.net GmbH, Ulm, Germany	5'-AGCTTTACCCTCCCATCCCT
PervCexc5.23_1r	biomers.net GmbH, Ulm, Germany	5'-TCAGGGATCGAACCTGCAAC
PervCexc5.23_2f	biomers.net GmbH, Ulm, Germany	5'-GTTCAGACCATCAGGGCTCC

PervCexc5.23_2r	biomers.net GmbH, Ulm, Germany	5'-GTCAGGGATCGAACCTGCAA
PervCexc5.23_3f	biomers.net GmbH, Ulm, Germany	5'-CTTCTGAATCTGGCAGGTAAGG
PervCexc5.23_3r	biomers.net GmbH, Ulm, Germany	5'-GTCTTCAGCCCAAGAAGTATGT
PervCexc5.23_4f	biomers.net GmbH, Ulm, Germany	5'-CTGACTCTGCTCCACCAATAG
PervCexc5.23_4r	biomers.net GmbH, Ulm, Germany	5'-ACCAGGCTCTTAAACCATCTC
PervCexc5.23seq1f	biomers.net GmbH, Ulm, Germany	5'-TGATACTCTTTTACAATTTTGGG
PervCexc5.23seq1r	biomers.net GmbH, Ulm, Germany	5'-AGACAACAGGAATGCTGAAGAAGGG
PervCexc1.20seq1f	biomers.net GmbH, Ulm, Germany	5'-CCAATGTATCCATGTAAATTTCCC
PervCexc1.20seq1r	biomers.net GmbH, Ulm, Germany	5'-GGGTGTGTGCAAAGGGGAGTGAG
PervCexc1.3seq1f	biomers.net GmbH, Ulm, Germany	5'-GCGTTTGGGAAGAGGGAGGG
PervCexc1.3seq1r	biomers.net GmbH, Ulm, Germany	5'-GCCAGCTTCAGCCTGGGC

Table 2 Primers used for establishing PCRs to detect PERV-C sites in the pig genome. Primers of optimized PCRs are marked in green when used with the cycler protocol "PERVC1" or marked in blue when eventually used with the cycler protocol "PERVC3". Primers located within the respective amplicons used for Sanger sequencing carry the abbreviation "seq".

Established PCR protocols and mastermix for the later used primer pairs:

95°C	5min	
94°C	30 sec	} 35x
58°C	30 sec	
72°C	2:15 mi	
72°C	10 min	
4°C	5 min	

Table 3 Cycler protocol "PERVC1" used for various primer pairs.

95°C	5min	
94°C	30 sec	} 35x
58°C	30 sec	
72°C	45 se	
72°C	10 min	
4°C	5 min	

Table 4 Cycler protocol "PERVC3" used for two primer pairs.

number of samples	1	5
H_2O	14	70
dNTPs	2	10
10x CoralLoad PCR buffer	2	10
MgCl	0	0
For primer	0,4	2
Rev primer	0,4	2
HST Taq	0,2	1

Table 5 Mastermix for PCRs with HotStarTaq Plus DNA Polymerase.

Sanger sequencing was done according to the established protocol in our laboratory. PCR products were extracted from blocks of agarose that have been excised from electrophorese gels by a scalpel under UV-light control by using either the "Double Pure Kombi Kit" (Bio&Sell GmbH, Feucht/Nürnberg, Germany) or "NucleoSpin® Gel and PCR Clean-up" (Macherey-Nagel, Düren, Germany). The chain termination reaction was performed by using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Waltham, Massachusetts, USA) (**Table 6** and **Table 7**). Cleanup of the sequencing reaction was performed by high-salt/Ethanol precipitation (**Table 8**). Capillary electrophoresis of the purified sequencing reactions was performed at the Helmholtz Center Munich (Neuherberg, Germany).

number of samples+2	
	x 4µl 5xSequencing Buffer
	x 1µl BigDye
	x 1µl Primer (10µM Stock)
	x 2µl H2O
	+ 2µl Template

Table 6 Composition of Sanger sequencing reaction.

95°C	1 min	
95°C	10 sec	} 40 x
54°C	10 sec	
60°C	4 mi	
4°C	15 min	

Table 7 Cycler protocol of the Sanger sequencing reaction.

Always fresh preparing of precipitation mix: number of samples + 5 x 10µl H2O x 5µl 125mM Ethylendiamintetraazetat (EDTA) x 60µl 100% Ethanol (EtOH) = total amount
Mix each sequencing sample with 75µl of precipitation mix and transfer to a new, labelled 1.5ml Eppendorf tube
Let it incubate on ice for 15 minutes
Centrifugate at 13000 rounds per minute (rpm) at 4°C for 30 minutes
Pay attention to the direction in which the Eppendorf tubes were placed into the centrifuge, because of the pellet, which will be there on the ground of the tube
Take off the supernatant carefully with two pipet tips, without filter, placed on top of each other
Afterwards, wash the pellet with 70% EtOH, which means shortly vortexing the pellet with the EtOH on top of it
Then, centrifugate again at 13000 rpm for 2,5 minutes
Take off the supernatant EtOH with the two-pipet tip technique
Let the pellet air dry for exactly 6 minutes
Resolve again in 30µl H_2O, vortex shortly, spin down in the centrifuge for a few seconds afterwards and transfer to 96-well sequencing plate

Table 8 Clean-up protocol of the Sanger sequencing reaction.

Sequencing data were analyzed as electropherograms by using FinchTV v.1.3.1/ FinchTV v.1.4.0 software and aligned compared to the corresponding sequences from the SusScrofa reference genome 11.1 (www.ensembl.org) in BioEdit.

Materials used in the laboratory and stable to acquire and process the samples:

product	**manufacturer**
Thermocycler GeneAmp® PCR System 9700	Applied Biosystems™, Waltham, Massachusetts, USA
Gel Doc 2000	BioRad, Hercules, California, USA
power supply unit Powerpack 300	BioRad, Hercules, California, USA
Thermoshaker TS100 / Block SC-24n	bioSan, Riga, Lettland
PCR Strips of 8 caps	Brand, Wertheim, Germany
PCR Strips of 8 tubes	Brand, Wertheim, Germany
Microwave Severin 900	Severin, Sundern, Germany
Eppendorf Centrifuge 5424	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Pipet tips epT.I.P.S.® Standard, Eppendorf Quality™, 2 – 200 µL, 53 mm, yellow	Eppendorf, Hamburg, Germany
Pipet tips epT.I.P.S.® Standard, Eppendorf Quality™, 50 – 1000 µL, 71 mm, blue	Eppendorf, Hamburg, Germany
Pipet tips epT.I.P.S.® Standard, Eppendorf Quality™, 0.1 – 10 µL S, 34 mm, dark grey	Eppendorf, Hamburg, Germany
Pipets Gilson (P2, P10, P20, P100, P200, P1000)	Gilson Inc., Middleton, WI 53562-0027, USA
Water bad JBN 5	Grant Instruments LTD, Shepreth, Royston SG8 6GB, UK
Pipet tips A20S	Kisker, Steinfurt, Germany
Pipet tips A200S	Kisker, Steinfurt, Germany
Pipet tips A1000S	Kisker, Steinfurt, Germany

Pipet tips A300SX	Kisker, Steinfurt, Germany
Pipet tips A100S	Kisker, Steinfurt, Germany
Rotina 380 R	Hettich Zentrifugen, Tuttlingen, Germany
Microcentrifuge	CarlRoth, Karlsruhe, Germany
Labcycler Basic	SensoQuest, Göttingen, Germany
Labcycler Gradient	SensoQuest, Göttingen, Germany
Sony UP-895CE (photo printer)	Sony, Minato, Tokio, Japan
OWL EASYCAST™ B2 (gel chamber with tray and combs)	ThermoScientific, Waltham, Massachusetts, USA
OWL EASYCAST™ A1B (gel chamber with tray and combs)	ThermoScientific, Waltham, Massachusetts, USA
VWR Labdancer	VWR International GmbH, Darmstadt, Germany
MK-2000B (scale)	Chyo, Japan
Spectrometer SimpliNano with printer	GE Healthcare Life Sciences, UK
Safe-Lock Tubes (1,5 ml, 2 ml, 5 ml)	Eppendorf, Hamburg, Germany
Measuring cylinder Labsolute®	Th. Geyer, Renningen, Germany
Erlenmeyer flask Simax®	Kavalierglass, 285 06 Sázava, Czech Republic
glasbottles Duran® (1l, 500ml, 250ml)	DWK Life Sciences, Wertheim, Germany
Autoklav Varioklav 400	HP Medizintechnik, Oberschleißheim, Germany
gloves SafeGrip	Süd-Laborbedarf, Gauting, Germany
Ethanol ROTIPURAN ® ≥99,8%, p.a.; Art.-Nr. 9065.4 (EtOH)	CarlRoth, Karlsruhe, Germany
Agarose universal; Art.-Nr. BS20.46.500	BIO&SELL, Feucht/Nürnberg, Germany
Tris Pufferan ≥99,9%, p.a.; 2M; Art.-Nr. 4855.2	CarlRoth, Karlsruhe, Germany

EDTA ≥99% 50mM, p.a., ACS; Art.-Nr. 8043.2	CarlRoth, Karlsruhe, Germany
100 mM dNTP Set, PCR Grade	Invitrogen, Karlsruhe, Germany
GeneRuler 1 kb DNA Ladder	ThermoScientific, Waltham, Massachusetts, USA
GELRED 10000x in water	Biotium, Fremont, CA 94538, USA
Bromophenol blue sodium salt for electrophoresis; Art.-Nr. A512.1	CarlRoth, Karlsruhe, Germany
Dry ice	AirLiquid, France
Trichloromethane/chloroform ROTIPURAN® ≥99 %, p.a. (CHCl3)	CarlRoth, Karlsruhe, Germany
caustic soda 5 mol/l (NaOH)	CarlRoth, Karlsruhe, Germany
DNeasy® Blood&Tissue Kit	Qiagen, Hilden, Germany
Nexttec™ 1-Step Tissue & Cells DNA isolation Kit	Nexttec™ Biotechnologie GmbH, Leverkusen, Germany
NucleoSpin® Gel and PCR Clean-up (250 preps)	Macherey-Nagel, Düren, Germany
HotStarTaq Plus DNA Polymerase	Qiagen, Hilden, Germany
Herculase II Fusion DNA Polymerase	Agilent, USA
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems™, Waltham, Massachusetts, USA
Double Pure Kombi Kit	Bio&Sell GmbH, Feucht/Nürnberg, Germany
Tailcropping-transfomer 230 V („Schwanzkupiertrafo “)	BEG Schulze Bremer GmbH, Dülmen-Rorup, Germany

Programmes used:

name of the programme	**provider**
BioEdit Sequence Alignment Editor 7.0.5.2/7.0.5.3	BioEdit
FinchTV v.1.3.1/ FinchTV v.1.4.0	Geospiza Inc.
Quantity One	Bio-Rad
Jalview (WATERHOUSE et al., 2009)	www.jalview.org

4 Results

During the preparation of my doctoral thesis, I worked on the multi-genetically modified breeding herd of donor pigs for xenotransplantation. The main focus of my work was laid in the examination of the hygiene status of the existing xenotransplantation donor herds at the Lehr- und Versuchsgut Oberschleißheim (LVG) and the sanitation of the hygiene status during the population of CiMM. This included exogenous as well as endogenous pathogens. This work was accompanied by the management of the existing donor herds at the LVG and the production of multi-modified piglets for pre-clinical research (**Figure 1**). Imbedded in the CRC TRR 127 there are two projects that I supplied with pigs. The C8 project managed by Prof. Reichart performing the pig-to-baboon xenotransplantation trials (LANGIN et al., 2018) (**Table 9**) and the C3 project, conducted by Prof. Seissler transplanting neonatal pig islet-like cell clusters, expressing LEA29Y into diabetic mice (KLYMIUK et al., 2012a; BUERCK et al., 2017) (**Table 10**). During my thesis I contributed to 4 accepted publications, on one of them I am the sole first author (see 4.1 – 4.4). In addition, I worked on the examination of PERV-C proviral integration sites (see 4.5).

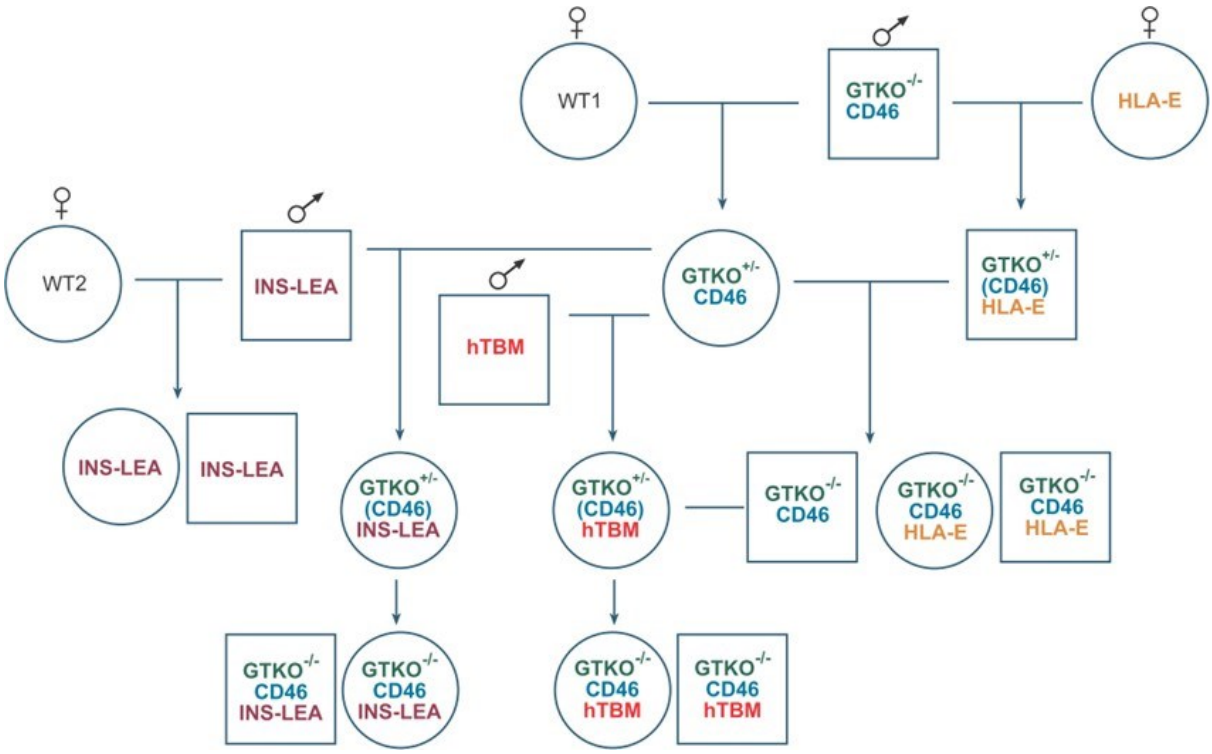

Figure 1 Establishing multi-modified donor pigs for xenotransplantation by combinatorial breeding. (provided by N.Klymiuk)

boar	sow	date	piglets	desired genotyp	used in C8
4667	4775	28.02.2017	11	**8x GTKO.hCD46.hTBM**	**4x --> C8**
5001	4776	20.07.2017	7	**2xGTKO.hCD46.hTBM**	**2x --> C8**
5001	5022	02.08.2017	6	3xGTKO.hCD46.hTBM	/
5001	5297	04.09.2017	8		/
5001	4775	26.09.2017	6	**1xGTKO.hCD46.hTBM**	**1x --> C8**
5003	5160	28.09.2017	6	**4xGTKO.hCD46.hTBM**	**2x --> C8**
5154	4672	24.10.2017	10	4xGTKO.hCD46.hTBM	/
5155	5295	14.12.2017	3	1xGTKO.hCD46.hTBM	/
5154	4776	18.12.2017	5	**5xGTKO.hCD46.hTBM**	**5x --> C8**
5001	5022	22.12.2017	4		/
5154	5019	16.01.2018	13	8xGTKO.hCD46.hTBM	/
5154	5426	26.01.2018	2	2xGTKO.hCD46.hTBM	/
5001	5160	01.03.2018	8	1xGTKO.hCD46.hTBM	/
5001	4775	15.03.2018	9	4xGTKO.hCD46.hTBM	/
WT	4776	02.08.2018	7		/
5411	5426	20.07.2018	3	2xGTKO.hCD46.hTBM	/
5001	4775	02.08.2018	8	3xGTKO.hCD46.hTBM	/
5411	5637	10.08.2018	3	**3xGTKO.hCD46.hTBM**	**3x --> C8**
5001	5295	10.10.2018	7	**2xGTKO.hCD46.hTBM**	**2x --> C8**
5625	5700	31.10.2018	7	2xGTKO.hCD46.hTBM	/
5001	5160	02.11.2018	6		/
5625	5806	28.11.2018	9	**5xGTKO.hCD46.hTBM**	**3x --> C8**
5001	4776	17.12.2018	3	1xGTKO.hCD46.hTBM	/
5001	5637	09.03.2019	14	**2xGTKO.hCD46.hTBM**	**2x --> C8**
5625	5700	28.03.2019	9	**2xGTKO.hCD46.hTBM**	**2x --> C8**
5411	WT	13.06.2019	13		/
5001	6087	07.07.2019	4	1xGTKO.hCD46.hTBM	/
5411	5019	11.08.2019	4	**2xGTKO.hCD46.hTBM**	**2x --> C8**
5625	5700	21.08.2019	9	**5xGTKO.hCD46.hTBM**	**3x --> C8**

Table 9 All matings and offspring generated for the C8 project of the CRC TRR 127.

boar	sow	date	piglets	desired genotypes			used in C3
4682	5019	09.07.2017	9	8xGTKO			/
5003	5160	28.09.2017	6				/
5003	5297	16.03.2018	9	**1xGTKO**	**3xGTKO.bLEA**	**2xGTKO.hCD46.bLEA**	**3x --> C3**
5003	5019	13.06.2018	9	**2xGTKO**	**1xGTKO.bLEA**	**3xGTKO.hCD46.bLEA**	**4x --> C3**
5003	5297	17.08.2018	8	**1xGTKO**	**3xGTKO.bLEA**	**4xGTKO.hCD46.bLEA**	**3x --> C3**
5625	WT	10.01.2019	12				/
5003	5019	08.02.2019	12	**1xGTKO**	**1xGTKO.bLEA**	**3xGTKO.hCD46.bLEA**	**/**
5626	5297	08.02.2019	9			**4xGTKO.hCD46.bLEA**	**/**
5626	WT	14.06.2019	10				
5626	5297	01.07.2019	2				

Table 10 All matings and offspring generated for the project C3 of the CRC TRR 127.

4.1 Population and raising up the xenotransplantation herd at CiMM

In the manuscript “Early weaning completely eliminates porcine cytomegalovirus from a newly established pig donor facility for xenotransplantation” we document the ability to sanitate the hygiene status of an existing herd comprising multiple genetic modification by populating a new facility with pregnant sows from a barrier facility, the motherless raising of their offspring and the usage of the female offspring as recipients of genetically modified embryos. I contributed personally by aiding the veterinary herd management of both the embryo transfer recipient as well as the genetically modified offspring; by taking blood, stool and nasal samples and the orchestration of their analysis; by co-writing of the manuscript.

(The supplementary table S1 can be found in the appendix)

The approved manuscript can be found at *Xenotransplantation. 2018 Jul;25(4):e12449. doi: 10.1111/xen.12449.*

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Xenotransplantation

ORIGINAL ARTICLE

Early weaning completely eliminates porcine cytomegalovirus from a newly established pig donor facility for xenotransplantation

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ABSTRACT

For clinical xenotransplantation, transplants must be free of porcine cytomegalovirus (PCMV). Piglets become infected primarily in the perinatal period by the mother sow. While individual donor animals can be protected from infection by isolation husbandry, success is not guaranteed and this strategy poses the risk of undetected infections and raises animal welfare questions. Here, we present the establishment of a completely PCMV- negative pig herd for breeding donor animals for xenotransplantation. Eleven pregnant DanAvl Basic

hybrid sows were purchased from a designated pathogen- free (DPF), PCMV- positive colony and transferred to a new pig facility at the Centre for Innovative Medical Models (CiMM) 4 weeks prior to farrowing. At the age of 24 hours, piglets were early- weaned and transferred to a commercially available Rescue Deck system dedicated to motherless rearing of piglets. Sows were removed from the facility. The PCMV status of F1- generation animals was determined at regular intervals over a period of 14 months by a sensitive real- time PCR-based detection method testing blood, nasal swabs and cultured peripheral blood mononuclear cells (PBMCs). F1 sows were used as recipients of genetically modified embryos to generate a xenotransplant donor herd. Offspring were tested for PCMV accordingly. All offspring have remained PCMV negative over the whole observation period of 14 months. A completely PCMV- negative pig herd for xenotransplantation has thus been successfully established.

KEYWORDS
early weaning, pig-to-human xenotransplantation, porcine cytomegalovirus, virus safety

1. INTRODUCTION

With recent successes in overcoming the major immunological hurdles in xenotransplantation[1-4], the goal of reaching clinically feasible pig-to-human transplantation has become imminent. With this, the second great obstacle, the microbiological safety of potential donor tissues and organs, demands attention. After organ allotransplantation, opportunistic exogenous viral infections are rare, but happen, as the antiviral immune response is diminished by immunosuppression. Over almost 20 years, this issue has been discussed in the field of xenotransplantation and, over time, a number of consensus papers and pathogen lists have been published regarding potentially harmful microorganisms in the context of xenotransplantation[5-7]. Additionally, transmission of pathogens with viable cells poses a very efficient direct infection route via the transplant[8]. In a very recent publication, Fishman[9] draws an updated list of pathogens that may be considered in the development of a screening program for xenotransplant donor pigs.

While most of these pathogens are sufficiently controlled by housing animals within barrier facilities and thus protecting them from exogenous infections, porcine endogenous retroviruses (PERVs) are a more precarious issue as they can be transmitted via the germline. Consequently, PERVs have caused considerable concern for clinical xenotransplantation. But to date there has been no report of PERVs being actually transmitted to the recipient of a xenograft or having caused detectable adverse reactions within a transplant[10]. Recent successes in eliminating PERVs from the genome of pig lines have further diminished this potential hazard[11,12]. Other pathogens, however, provoke lifelong, latent, transmittable infections, and are prevalent in most, if not all, pig populations and are thus difficult to control. These include the porcine cytomegalovirus (PCMV), a β-herpesvirus related to the human cytomegaloviruses that causes systemic disease and potentially leads to graft failure in human allotransplantation[13]. In xenotransplantation, the PCMV has also been associated with transplant injury. This has been largely attributed to virus activation within the graft following transplantation but initially was not thought to cause invasive disease in the recipient[14,15]. It is still unclear whether PCMV can infect human cells, with one in vitro study suggesting the possibility[16] and a different study presenting evidence for the opposite[17]. There are indications that while in vitro PCMV appears susceptible to standard antiviral medication comparable to that employed in allotransplantation[18], in vivo data from pig-to-baboon xenotransplantation suggest that the commonly used ganciclovir has no therapeutic efficacy against PCMV at standard doses[19]. Agents that do prevent or treat PCMV infection effectively, such as foscarnet or cidofovir, carry significant toxic potential for the transplant recipient and are thus of limited usefulness. Consequently, there is consensus that potential donor animals for xenotransplantation should be free of PCMV.

Pigs are mostly infected with PCMV in the perinatal period by the mother sow or postnatally through oronasal secretions of virus shedding animals[20]. The virus is endemic in pig herds worldwide[20,21]. To avoid transmission of virus from infected mother sows to offspring, contact between sows and piglets has to be minimized. With strategies of early weaning within the first 2 weeks after birth and separate rearing of early-weaned piglets, it has previously been possible to generate individual PCMV-negative pigs as organ donors[20-24]. However, success was not reliably predictable and came at the immense effort of having to raise all potential donor animals motherless in isolation.

In this study, we report on the design and implementation of a strategy of very early weaning to generate a completely PCMV-free donor breeding herd for xenotransplantation in a newly established pig facility for biomedical research at the Centre for Innovative Medical Models (CiMM), LMU Munich, Germany. We show that with weaning at an age of 24 hours and subsequent immediate removal of all PCMV-positive mother sows from the facility, we have been able to generate a herd of PCMV-negative sows. In a subsequent step, we utilized these sows as recipients for embryo transfer to introduce all our genetically modified (gm) pig lines for xenotransplantation into the facility. All recipient sows and all gm offspring have stayed PCMV negative over the total observation period of 14 months. To our knowledge, this is the first report of a completely PCMV-negative pig facility that allows for conventional breeding of donor animals for xenotransplantation without further need for separate rearing of individual animals in isolation.

2. MATERIAL AND METHODS

2.1 Ethics Statement

All animal work was performed with the permission of the local regulatory authority, Regierung von Oberbayern (ROB), Sachgebiet 54, 80534 München (approval number: 55.2-1-54-2532.0-82-2016). Applications were reviewed by the ethics committee according to §15 TSchG (German Animal Welfare Act).

2.2 Populating CiMM

For establishing the breeding herd for xenontransplantation, eleven pregnant PCMV-positive DanAvl basic hybrid sows were purchased from a designated pathogen-free (DPF) barrier facility (Vermarktungsgemeinschaft für Zucht- und Nutzvieh e.G., Fehmarn Hof Schweinehaltungs KG, Fehmarn, Germany) located on the island of Fehmarn in the Baltic Sea and introduced into CiMM 4 weeks prior to their farrowing date (Figure 1). These sows were the founder population for CiMM (F0 generation). They were screened for PCMV 28 days before and 1 day after farrowing (Table 1). On gestation day 114, birth was induced by intramuscular administration of 0.175 mg cloprostenol (Estrumate®, Intervet GmbH, Unterschleissheim, Germany). After birth, offspring (F1 generation) were separated from the mother sow immediately and held in groups in isolation boxes under infrared light. Once every 2 hours, piglets were allowed to suckle colostrum under supervision to prevent oronasal

contact between sows and their litters. After 24 hours, all F0 sows were removed from CiMM and the 91 F1 piglets were weaned to a motherless rearing system (Rescue Deck, Provimi Rescue Decks). Here, they were raised with designated milk replacers (Bonimal SB Powermilk, BayWa, Germany) and mash feed (Bonimal SB Liquidstart 2.0, BayWa, Germany) over a period of 3 weeks after which they were transferred to the regular holding pens within the facility (Figure 1). All F1-generation animals were screened for PCMV at different time points over a total time period of 14 months (Table S1). Upon reaching sexual maturity, PCMV-negative F1 sows were utilized as recipients for embryo transfer to introduce gm-modified pig lines into CiMM. Embryos were produced by either somatic cell nuclear transfer (SCNT) [25] or in vitro fertilization (IVF) [26]. For SCNT, pig primary kidney cells were isolated from existing gm pig lines[27] and used as nuclear donor cells. For IVF, epididymidal sperm was collected from gm boars to fertilize oocytes isolated from slaughterhouse ovaries. SCNT and IVF embryos were treated with 0.25% trypsin according to the International Embryo Technology Society (IETS) embryo-treatment protocol[28] and transferred to the estrus-synchronized recipient sows (Figure 2).

Gm-modified offspring farrowed from the PCMV-negative F1 sows were then tested for PCMV at one single time point (Table 2).

2. 3 In vivo sampling of pigs

Blood sampling from adult sows was performed without sedation under manual fixation. Whole blood was drawn from the jugular vein with single-use needles (Ehrhardt Medizinprodukte, Geislingen, Germany) into lithium heparin and serum Monovettes® (Sarstedt, Nümbrecht, Germany). Nasal swabs were taken during the same procedure by inserting sterile dry swabs into one nostril, without touching outer skin, to collect nasal mucosa and capillary blood (Henry Schein, Hamburg, Germany). Lithium heparin blood and swabs were then cooled to 4°C and used freshly for analysis. Serum was centrifuged at 6°C, 1800 *g* for 10 minutes, then aliquoted to 1mL samples and stored at -80°C. For piglets, all blood sampling was performed accordingly. Oral swabs were taken during the first sampling procedure at an age of 1 day. For this, similar swabs as employed for nasal probing were inserted into the mouth of the piglets to collect mucous membrane from the palate.

2. 4 Ex vivo sampling of pigs

ET-recipient F1 sows were euthanized under ketamine (Ursotamin®, Serumwerk Bernburg, Germany) and azaperone (Stresnil®, Elanco Animal Health, Bad Homburg, Germany) anesthesia by intravenous injection of T61® (Intervet GmbH), according to the manufacturer's instructions. Whole blood was drawn directly from the heart, and the following organs were sampled: liver, lung, heart, pancreas, spleen, kidney, and lymph nodes. Small pieces of about 5x5x5 mm in size were excised from each organ and frozen to the core by placing them on dry ice plates. They were then transferred to pre-cooled Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored at -80°C. Whole blood was aliquoted in 1mL Eppendorf tubes and also stored at -80°C. Organ samples from stillborn, dead, or euthanized offspring were collected as described above. For these animals, sampling of liver, spleen, whole blood, serum, and, if possible, bile was performed. All samples were stored at -80°C until analysis.

2. 5 Screening Strategy

Blood and nasal swabs of F0 sows were tested for PCMV 28 days before and 1 day after farrowing (Table 1). All 91 F1 piglets were screened repeatedly over a period of 14 months for the presence of PCMV (Table S1). Nineteen piglets born from these animals were tested at one time point when piglets were between 14 and 30 days old (Table 2). Additionally, organs of stillborn, dead, or euthanized piglets were sampled and examined for PCMV infection.

Regular hygiene monitoring is performed, and representative numbers of the present pig population within the facility are examined for the presence of a range of pathogens on serological and antigenic level. Serological testing for Actinobacillus pleuropneumoniae, Haemophilus parasuis, Lawsonia intracellularis, Leptospira spp., Mycoplasma hyopneumoniae, Pasteurella multocida, porcine reproductive and respiratory syndrome virus, swine influenza virus, transmissible gastroenteritis, and hepatitis E virus, as well as antigen testing for Brachyspira hyodysenteriae, Lawsonia intracellularis, salmonella and swine influenza virus, and evaluation of bacteriological and endoparasitic content of feces, is performed on serum, EDTA whole blood, and fecal samples by a commercial laboratory (Vaxxinova GmbH, Münster, Germany) employing their standardized ELISA- and PCR- based test systems.

2. 6 Peripheral blood mononuclear cell (PBMC) isolation and cultivation

Porcine PBMCs were isolated by Ficoll gradient centrifugation (lymphocyte separation medium, PromoCell, Heidelberg, Germany), using Falcon tubes without porous barriers. The isolated PBMCs were washed and cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) (Merck Millipore, Darmstadt, Germany). DNA was isolated before and after incubation.

2. 7 DNA extraction

DNA was extracted from blood, purified PBMCs, nasal and oral swabs, or tissues using DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). DNA was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Worcester, MA, USA).

2. 8 Real-time PCR

PCMV-specific real-time PCR testing was performed as described[15,29,30] using specific primers PCMV real fw 5' ACTTCGTCGCAGCTCATCTGA, PCMV real rev 5' GTTCTGGGATTCCGAGGTTG, and PCMV probe FAM- 5' CAGGGCGGCGGTCGAGCTC –BHQ[15]. To improve PCMV detection in blood or in purified PBMCs, PBMCs were incubated in culture medium previously shown to increase PCMV expression[29] and PCR analysis was performed twice, before and after incubation of PBMCs in culture medium. Detection limit of the real-time PCR was two-to-five copies of PCMV and was performed using a SensiFast Probe No ROX One-step Kit, according to supplier recommendations (Bioline GmbH, Germany). Sixty ng of DNA was used for testing. The reaction mixture contained 400 nmol/L of both primers and 100 nmol/L of the probe in a final volume of 20 µL. The following conditions for amplification were used: denaturation at 95°C for 5 minutes, 45 cycles of amplification with denaturation at 95°C for 10 seconds, annealing at 59°C for 20 seconds, and extension at 60°C for 25 seconds. Reporter fluorescence was measured using an Mx3005P Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA).

3. RESULTS

3.1 Establishment of the herd

In this study, we aimed at establishing a PCMV-free breeding herd of gm pigs for xenotransplantation. Pregnant F0 sows were purchased from an external designated pathogen-free barrier facility and introduced into CiMM (Figure 1). CiMM was opened in December 2016 as a newly built pig barrier facility. Quarantine requirements of 48 hours without outside pig contact before entry are in place. Cleanroom showers are used, and a full change of clothes is performed upon entering the facility. Schönhammer ventilation system was installed (Schönhammer, Mengkofen, Germany) to discharge pollutants and odors from the stable, target temperature stability and control humidity levels to prevent precipitation. F0 sows were the first pigs to enter this facility. In addition to PCMV screening, all animals were examined for the presence of the following pathogens on a serological and/or antigen basis: Serological testing was performed for Actinobacillus pleuropneumoniae, Haemophilus parasuis, Lawsonia intracellularis, Leptospira spp., Mycoplasma hyopneumoniae, Pasteurella multocida, porcine reproductive and respiratory syndrome virus, swine influenza virus, transmissible gastroenteritis, and hepatitis E virus. In addition, antigen testing took place for Brachyspira hyodysenteriae, Lawsonia intracellularis, salmonella and swine influenza virus, and fecal swabs were examined for bacteriological content and endoparasites. All testing is repeated continuously every 6 months on a representative proportion of the current pig population within CiMM to ensure adequate hygiene monitoring. To date, antigen detection of Brachyspira hyodysenteriae, Lawsonia intracellularis, and salmonella and swine influenza virus has remained negative. Serologically, the cohort is positive only for Actinobacillus pleuropneumoniae and Lawsonia intracellularis.

3.2 Screening for and elimination of PCMV

When testing for PCMV using a sensitive real-time PCR, seven of eleven F0 sows were at least once tested positive for PCMV (Table 1). Because of the premature death of sow 91107, no samples from this animal were available at the time of farrowing. When screening all 91 F1 piglets born from the F0 founder sows, we found no PCMV- positive animal at any time point over the whole observation period of 14 months. Detailed results of repeated PCMV screens are depicted in Table S1. Two F1 animals were euthanized at the age of 4 months for collection of a full- organ set, one born from a PCMV- positive (#13) and the other born from a PCMV-

negative mother sow (#27). As control, an age- matched pig from a cohort without early weaning was used (#5157). Both piglets derived from the early weaning procedure were shown PCMV negative in all tested organs (Figure 3).

Further, the gm offspring farrowed from the F1 PCMV-negative sows were tested for PCMV at one single time point, at which piglets were between 14 and 30 days old (Table 2). All 19 tested piglets were negative for PCMV, as were their respective mothers at the same time point. In addition, organ and blood samples were taken from stillborn, dead, or euthanized piglets and liver and spleen tissue was screened by real-time PCR for the presence of PCMV. All samples were tested negative, indicating the complete elimination of the virus from the breeding herd.

4. DISCUSSION

There is general consensus in the research community that potential donor animals must be free of PCMV if clinical xenotransplantation is to become reality. We present here the successful elimination of this virus from a complete breeding herd of gm pigs for biomedical research applications including xenotransplant donor animals. The pig population in this newly built and established research facility has remained free of detectable PCMV over the whole monitoring period of 14 months. This allows for the conventional breeding of experimental animals within the facility without having to employ measures such as isolated rearing of individual piglets as xenodonors.

Determination of the PCMV status in this study has been made via PCR-based detection of the virus genome. For testing, PCR-based methods[31] and immunologic methods detecting PCMV-specific antibodies[32] can be used. Furthermore, screening for PCMV using non-invasively taken samples was found effective[33]. Even though antibody titers have been reported from piglets infected very early in life[20], there is some evidence that no seroconversion takes place in piglets infected congenitally or neonatally[34], making serological testing ineffective in cases where such infection routes are suspected. We thus chose to attempt direct virus detection to examine successful elimination of PCMV from the breeding herd. Because PCMV titers can be low or even close to the detection limit[13], a PBMC culture system was established to promote virus replication and facilitate detection. As is demonstrated here, this cultivation step results in significantly higher virus titers in cells of animals that are positive for PCMV, reaching as far as providing positive results for animals

that on native blood samples had been below the detection limit (Table 1). Multiple testing of animals employing highly sensitive methods can thus be termed a necessity for confidently determining a PCMV-negative status within a pig cohort

Other research groups have given account of PCMV elimination by early weaning in piglets destined as donors for pig-to-primate transplantations[20-24]. Tucker et al [35] report that not in all cases has it been possible to retain all animals free of PCMV. This has been attributed to the possibility of transplacental fetal PCMV infections, as postulated by Edington et al[34] in 1977. In this work, intrauterine transmission of PCMV could be detected in fetuses of serologically negative mother sows that had become infected during pregnancy. Today, it is common sense that intrauterine PCMV infection of fetuses can take place. However, to our knowledge, there are no definitive reports of transplacental PCMV transmission to fetuses aside from experimentally induced infection in previously seronegative sows. While there seems to be indeed the chance of opportunistic infection due to first contact of sows with the virus during pregnancy, this appears not to be the case often. Moreover, piglets infected congenitally are usually weak and die within weeks of birth[21]. Consequently, early weaning programs for elimination of PCMV might more likely fail by accidental transmission of infectious material between different cohorts of isolated pigs through caretaker or research personnel than by congenital transmission of the virus. Our own findings of an albeit rather low number of PCMV-positive sows that nevertheless all gave birth to completely PCMV-negative offspring, support this notion. However, the incidence of inadvertent PCMV infections during previous attempts of generating PCMV-negative xenograft donors highlights the importance of barrier facilities completely devoid of the virus.

As PCMV is distributed by oronasal secretions and consequently airborne infection[36], all contact with potentially infectious discharge and aerosols must be avoided if transmission is to be excluded. That results in immense effort on the side of personnel coming into contact with the animals. Great care has to be taken when switching from one animal or animal cohort to the next. Typically, barrier animal housing facilities require individuals accessing the facility to follow some kind of quarantine procedure (eg, no contact to animals of the same species outside the facility within the past 48 hours) in addition to showering and complete change of clothes upon entering. Something similar would have to be employed within the facility if strict separation of individual pigs for infection protection is to be performed adequately, thus requiring such man power as can only be provided at enormous costs. PCMV sanitation of a

complete facility offers the great benefit of having to undertake complicated isolation measures only once as opposed to repeatedly protecting specific animals from PCMV infection by early weaning and rearing in isolators.

Finally, developments in recent years have shown that legislation concerning animal welfare has become ever stricter. This can surely be attributed to an increased perception of such issues within modern Western society that is not willing to tolerate unnecessary strain on experimental animals. The pig as a sociable animal falls under great stress if separated from its peers[37]. Isolation husbandry can thus already only be employed in exceptional cases, and the assessment of such cases is likely to become even more severe in the future. This is especially true if the procedure not only demands avoidance of direct physical contact between animals but also requires protection from the air space of other animals, meaning that it is difficult, if not impossible, to at least allow for visual and audio contact between pigs.

By eliminating PCMV completely from a whole pig research facility and thus allowing for animal production by conventional breeding, the grounds for regularly supplying xenotransplant donors have been strengthened. The cohort of xenopigs presented here may be utilized as a basis for deeper examination of their status regarding known and emerging infectious agents and subsequently improving the herd toward a supply chain for clinical xenotransplantation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

AB conceived and designed the facility sanitation concept. JD and UF conceived and designed the PCMV screening experiments. AB, SE, BK, VZ and MK performed the experiments for facility sanitation. UF performed the experiments for PCMV screening. AB, JD, UF, SE, BR, CK, NK, and EW drafted the manuscript. All authors read and approved the final manuscript.

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TABLE 1 PCMV screen of 11 Fehmarn mother sows

Time point	PPD[a] -28	PPD 0		PPD 0	
Sample	Blood	Nasal swab	Blood	PBMCs 5 d cultivation	PBMCs 7 d cultivation
#89030	–[b]	+[c]	–	–	–
#91106	+	+ +[d]	+	++	+ +
#91107	–	n.d.[e]	n.d.	n.d.	n.d.
#91108	+ +	–	–	+ +	+ +
#91111	–	–	+	+ +	+ +
#91112	–	–	–	–	–
#91114	–	–	–	–	–
#91117	+	–	–	+	+
#91118	–	–	–	–	–
#91119	–	+	+	+ +	+ +
#91122	+	–	–	–	+

[a]Postpartal day.
[b]Negative result.
[c]Positive result.
[d]Strongly positive result.
[e]Not determined.

All eleven DanAvl hybrid sows purchased from the barrier facility on Fehmarn were tested for PCMV 28 d prior to (PPD – 28) and 1d after farrowing (PPD 0) using sensitive real-time PCR. Seven of eleven F0 sows were tested positive for PCMV at least once. Because of the premature death of sow 91107, no samples from this animal were available at the time of farrowing. PBMC isolation and cultivation was performed from PPD 0 lithium heparin whole blood. PBMC cultivation increases virus production and detectability of PCMV.

TABLE 2 PCMV screen of genetically modified offspring

Pig	Mother sow	Day of litter	Age of piglet[a]	PCMV
#10001	79	Oct 30, 2017	30 d	–[b]
#10002			30 d	–
#10003			30 d	–
#10004			30 d	–
#10005	50	Nov 7, 2017	22 d	–
#10006			22 d	–
#10007			22 d	–
#10008	70	Nov 8, 2017	21 d	–
#10010	52	Nov 8, 2017	21 d	–
#10011			21 d	–
#10013			21 d	–
#10022	42	Nov 14, 2017	15 d	–
#10023			15 d	–
#10024			15 d	–
#10026			15 d	–
#10027			15 d	–
#10032	43	Nov 15, 2017	14 d	–
#10034			14 d	–
#10037			14 d	–

[a]All piglets were sampled Nov 28, 2017.

[b]Negative result.

Nineteen genetically modified offspring animals aged between 14 and 30 d and their respective F1- generation mother sows were tested negative for PCMV on cultivated PBMCs.

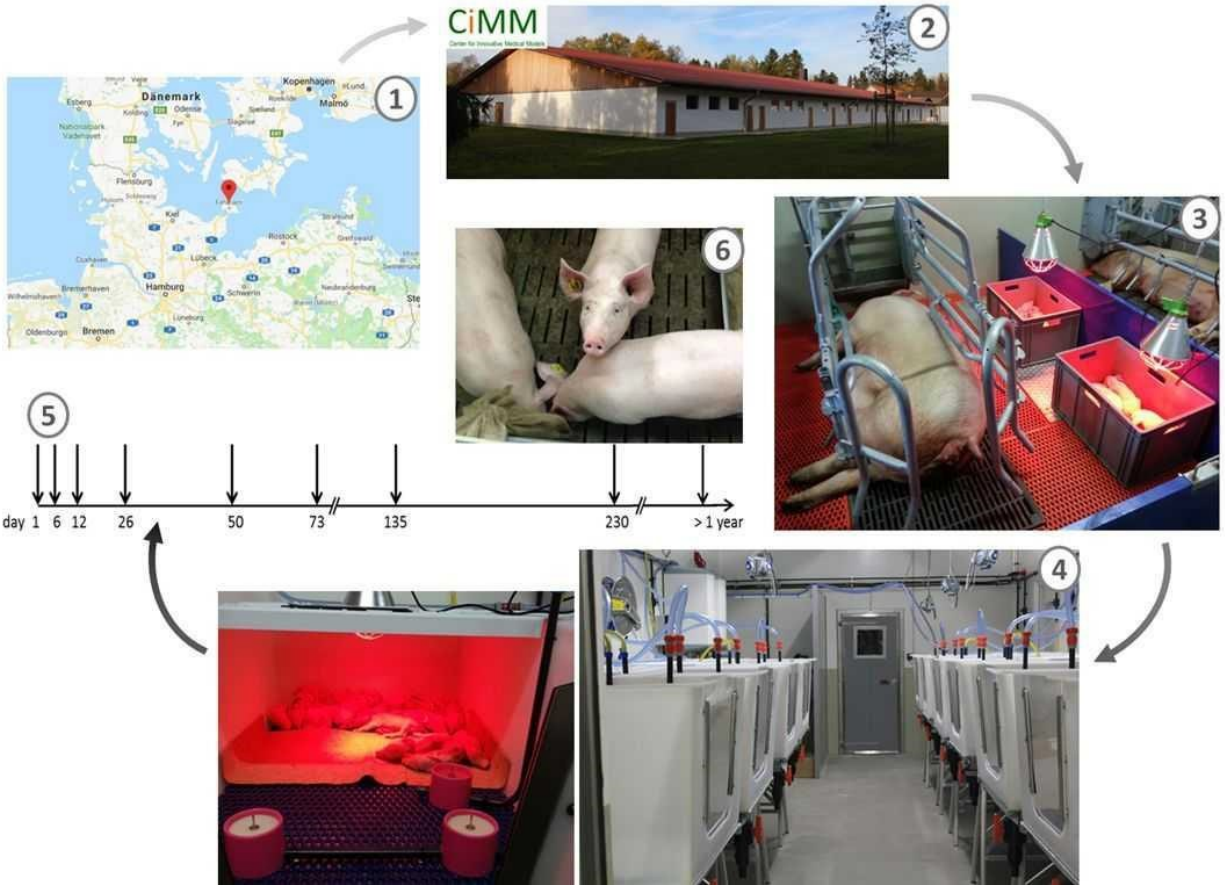

FIGURE 1 Establishment of CiMM. Eight pregnant PCMV-positive and 3 pregnant PCMV-negative DanAvl hybrid sows (F0) were purchased from a DPF barrier facility on the island of Fehmarn (1) and transferred to the newly established CiMM 4 wk prior to their farrowing date (2). Offspring (F1 generation) and sows were separated immediately after birth, and piglets were allowed to suckle colostrum under controlled conditions every 2 h for 24 h postpartum (3). Piglets were then transferred to a Rescue Deck system with milk replacer feeding, and sows were removed from the facility (4). F1 piglets were screened periodically for PCMV over a total period of 14 mo (5) and raised to serve as recipients for gm embryos (6).

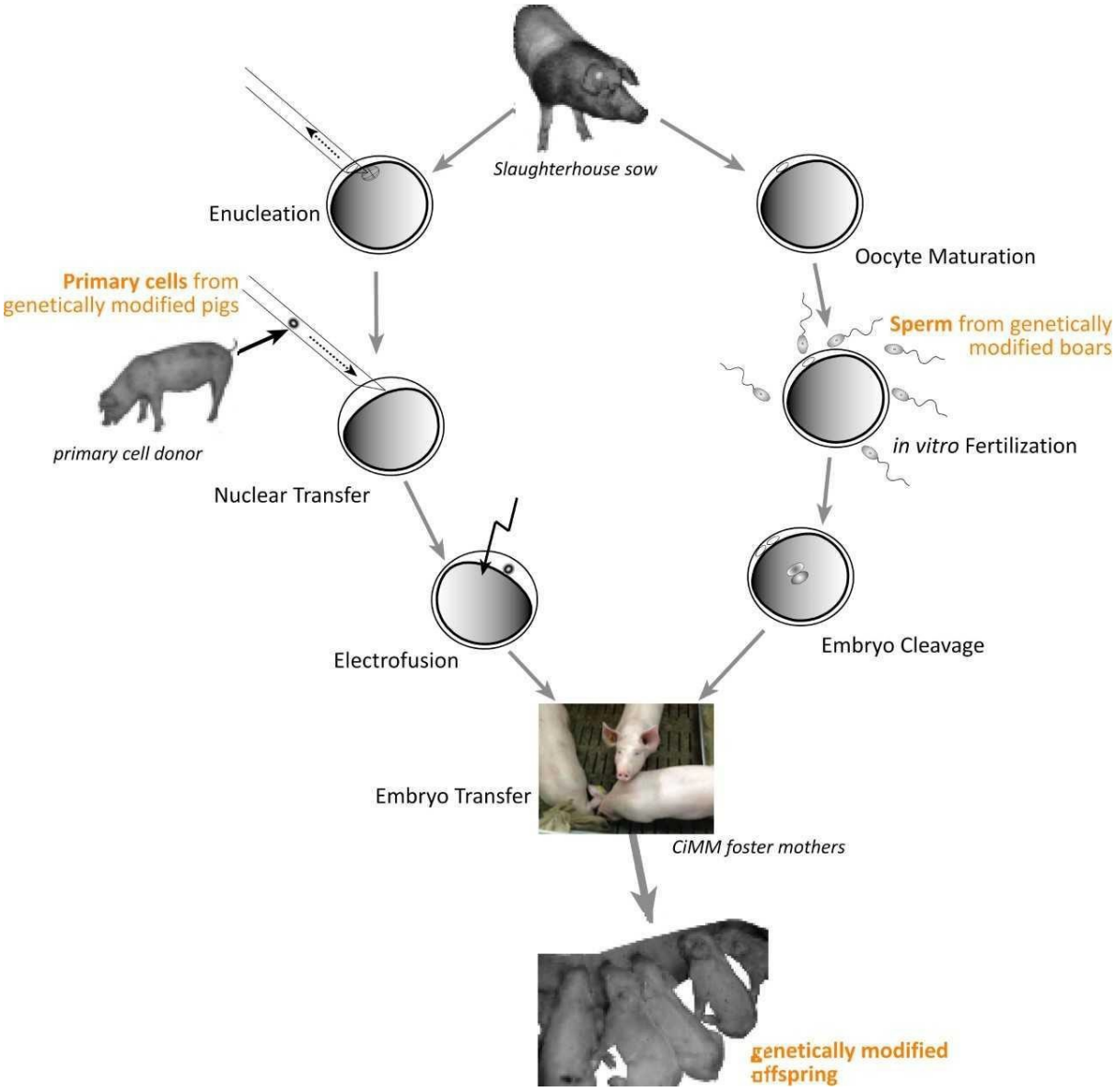

FIGURE 2 Establishment of PCMV negative xenograft donor herd. In vitro fertilization (IVF) was performed using in vitro matured (IVM) oocytes collected from slaughterhouse material. Genetically modified (gm) epididymal sperm collected from gm boars was used to fertilize the IVM oocytes. After fertilization, cumulus cells and excess sperm were removed and only oocytes with one or two visible polar bodies were used for embryo transfer to estrus-synchronized PCMV-negative F1 sows. For somatic cell nuclear transfer (SCNT), porcine oocytes were enucleated and gm porcine primary cells were injected into enucleated oocytes. After electric fusion and activation, embryos were transferred into the oviducts of estrus-synchronized PCMV-negative F1 sows.

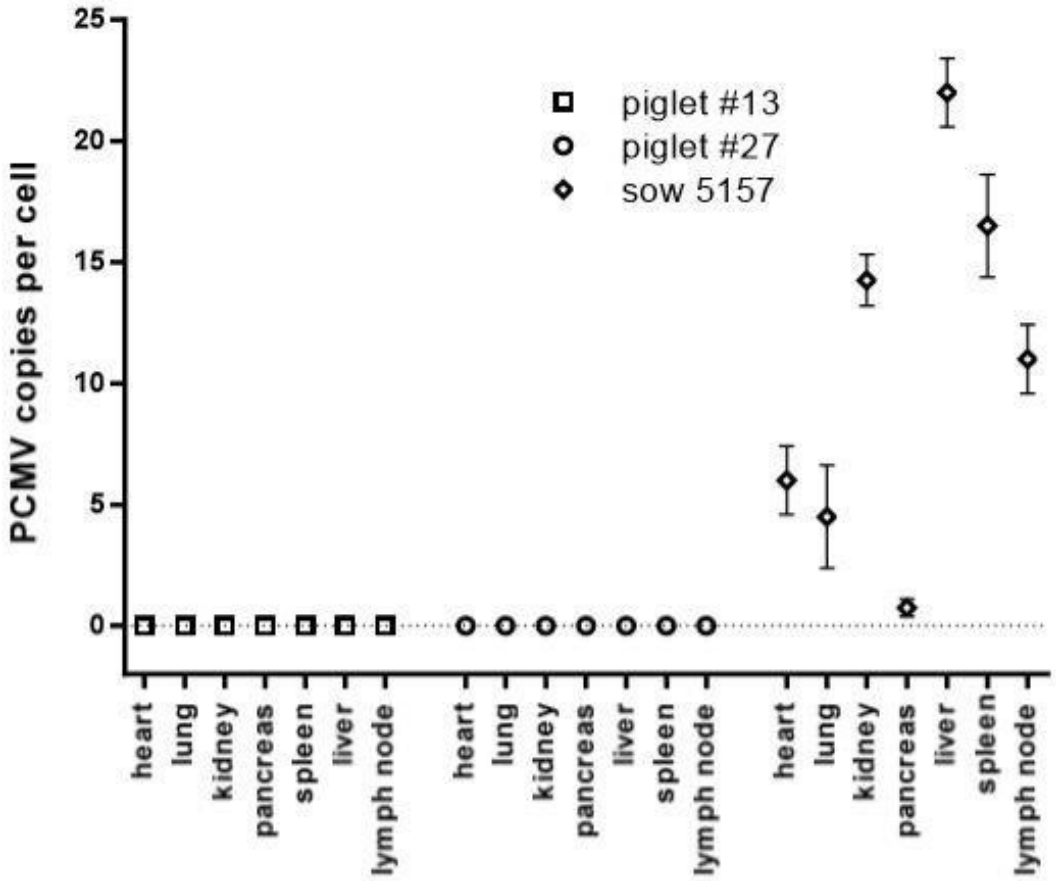

FIGURE 3 PCMV is eliminated from all organs of early weaned piglets.

Organ sets from two F1 animals were tested for PCMV. Piglet #13 was born from a PCMV-positive F0 mother sow, and piglet #27 was born from a PCMV-negative F0 mother sow. Both animals are negative for PCMV in all tested organs with zero copy numbers of PCMV detectable. Animal #5157 raised without early weaning in standard agricultural environment is clearly PCMV positive, with the highest copy numbers in liver tissue. Quantities are depicted as virus genome copy numbers per cell.

4.2 Consistent success in life-supporting porcine cardiac xenotransplantation

This article documents the long-time survival of baboon xenograft recipients after receiving a fully life-supporting heart from donor pigs that were raised and maintained at the Chair of Molecular Animals Breeding and Biotechnology (LANGIN et al., 2018). After decade-long struggling with orthotopic heart transplantations, three factors finally led to the success with the pig-to-primate xenotransplantation model. First, the genetic constellation of GTKO.hCD46.hTBM generated at the Chair for Molecular Animal Breeding and Biotechnology is obviously a simple but optimal basis for preventing antibody-mediated rejection and microthrombosis (MOHIUDDIN et al., 2016; SINGH et al., 2019). Second, the switch from the cold ischemic preservation (static preservation with 4°C cold crystalloid solutions, e.g. Custodiol®) to the non-ischemic preservation method developed by Steen et al. (STEEN et al., 2016) improved the weaning of the recipient baboons from the cardiopulmonary-bypass (CPB). Finally, another vital factor was the inhibition of the growth of the pigs' hearts in the recipients by a trinomial therapy approach: antihypertensive treatment for the baboons to match the blood pressure of the pigs, earlier weaning of cortisone and treatment with temsirolimus, the prodrug of sirolimus to attenuate heart overgrowth (LANGIN et al., 2018). My contribution to this work was the veterinary herd management of the pigs, the planning and scheduling of matings, the genotyping of offspring and the supply of healthy animals to the Walter-Brendel-Center for Experimental Surgery. The finally approved manuscript can be found at *Nature. 2018 Dec;564(7736):430-433. doi: 10.1038/s41586-018-0765-z.*

https://www.nature.com/articles/s41586-018-0765-z

4.3 Targeting αGal epitopes for multi-species embryo immunosurgery

In this paper we investigated whether serum of GTKO pigs is a sufficient and reliable source of anti-Gal antibodies for inducing complement-mediated lysis of the trophectoderm cells. In previous attempts, immuno-surgery had proven potential by first coating the trophectoderm with antibodies and second by lysing the trophectoderm by the complement system via the classical, antibody-mediated complement activating pathway. Blood serum should normally provide both, the antibodies against the embryo and the complement components. The

efficacy of the process, however, was unstable, depending on the serum used. The idea behind our approach was that most mammalian species carry αGal epitopes, whereas the few species that lack these epitopes develop high levels of antibodies against αGal as a result of permanent stimulation by αGal-carrying bacteria. Thus, it was evident to test the potential of serum from αGal-lacking individuals in immunosurgery. As GTKO animals have been developed at the Chair of Molecular Animal Breeding and Biotechnology, it was tempting to use their serum in such an approach. In the study, it was first examined, if the four mammalian species used in the study, mouse, rabbit, cattle and pig had αGal epitopes on their blastocysts, as it was only known for pig embryos, that αGal epitopes appear first at the 8-cell stage (CHI et al., 2012). Then, the αGal-antibody levels were determined in different GTKO animals. Finally, the concept of immunosurgery was confirmed by treating embryos from all three species with GTKO serum. With this approach the inner cell masses of blastocysts from all species could be isolated and though they were exposed to the complement serum before lysis, they showed satisfactory levels of purity. My contribution to this work was sampling of blood, preparation of serum, undertaking sections of the GTKO animals and preparation of samples for immunohistochemistry (IHC). The finally approved manuscript can be found at *Reprod Fertil Dev. 2019 Apr;31(4):820-826. doi: 10.1071/RD18120.*

https://www.publish.csiro.au/RD/RD18120

4.4 Transmission of porcine circovirus 3 (PCV3) by xenotransplantation of pig hearts into baboons

This manuscript described the first trans-species transmission of PCV3, as well as the abundance of PCV3 in our breeding herd. Due to its mild and ambiguous clinical course, PCV3 has been detected in pig herds only recently, but in the meanwhile, it turned out that the virus is pandemic in a significant proportion of herds all over the world. Evidently, it has been found in our existing xenotransplantation donor herd at LVG. In the xenotransplantation context it is important to consider that we here document the transmission of PCV3 to four baboons after transplantation of a heart from an infected donor pig. As PCV3 was found in all organs of the baboons, with even more virus load the longer the survival time of the baboon was, an active replication of the virus in the transplant and/or the recipient can be suggested. My

contribution was providing frequent blood samples of the pigs and coordination of sample analysis, as I could specify where exactly the animals were and so help investigation and discuss possible transmission and infection routes of PCV3 in our herd. Further investigations on the PCV3 transmission in our herd and further infection trials are currently ongoing. The approved manuscript can be found at *Viruses. 2019 Jul 16;11(7). pii: E650. doi: 10.3390/v11070650.*

https://www.mdpi.com/1999-4915/11/7/650

4.5Inheritance of porcine endogenous proviruses in the xenotransplantation breeding herd at MABB

One of the most critical characteristics of xenotransplantation donor animals is their status of PERV, particularly PERV-C. Ideally donor pigs should be free of PERV-C (DENNER et al., 2009). For clarifying the occupancy of the genomes of our xenotransplantation donor pig breeding herd, we followed two different strategies.

First, the porcine reference genome was examined for the PERV-A, -B and -C subfamilies. In total 24 PERV-A and 18 PERV-B were identified on the basis of their *env* gene sequences, but no PERV-C *env* was identified. On the basis of *LTR*-sequences, however, 69 PERV-A, 98 PERV-B and 25 PERV-C elements were found. Not all proviral-like structures contained a full provirus, rather they were regularly disrupted by larger gaps within the provirus or at one of their ends. In the latter case, they comprise only one single LTR and a determination of their age, based on the differences between the up- and downstream elements is not anymore possible. Unrooted phylogeny PERV LTR elements show a clear separation of the A-, B- and C-subfamilies (**Figure 2**). All up- and downstream pairs of LTR of given proviruses fall into the very same cluster. An interesting side-finding was that all proviruses with LTR clustering to the A-subfamily contained an *env* sequence similar or identical to the commonly used PERV-A reference virus (GenBank annotation AJ253656). In contrast, proviruses with LTR clustering to the B-subfamily) contained either *env* sequences similar or identical to the commonly used PERV B reference virus (GenBank no. AJ253657) or PERV-A env.

Most important for the PERV-C subfamily, however, was that many LTR clustered with the PERV-C reference virus (GenBank no. AF038600), but those comprised either solo-LTR or,

surprisingly, proviruses that lack large parts of the *env* gene. Thus, none of the proviruses in the SusScrofa reference genome 11.1 constituted a tropism of PERV-C.

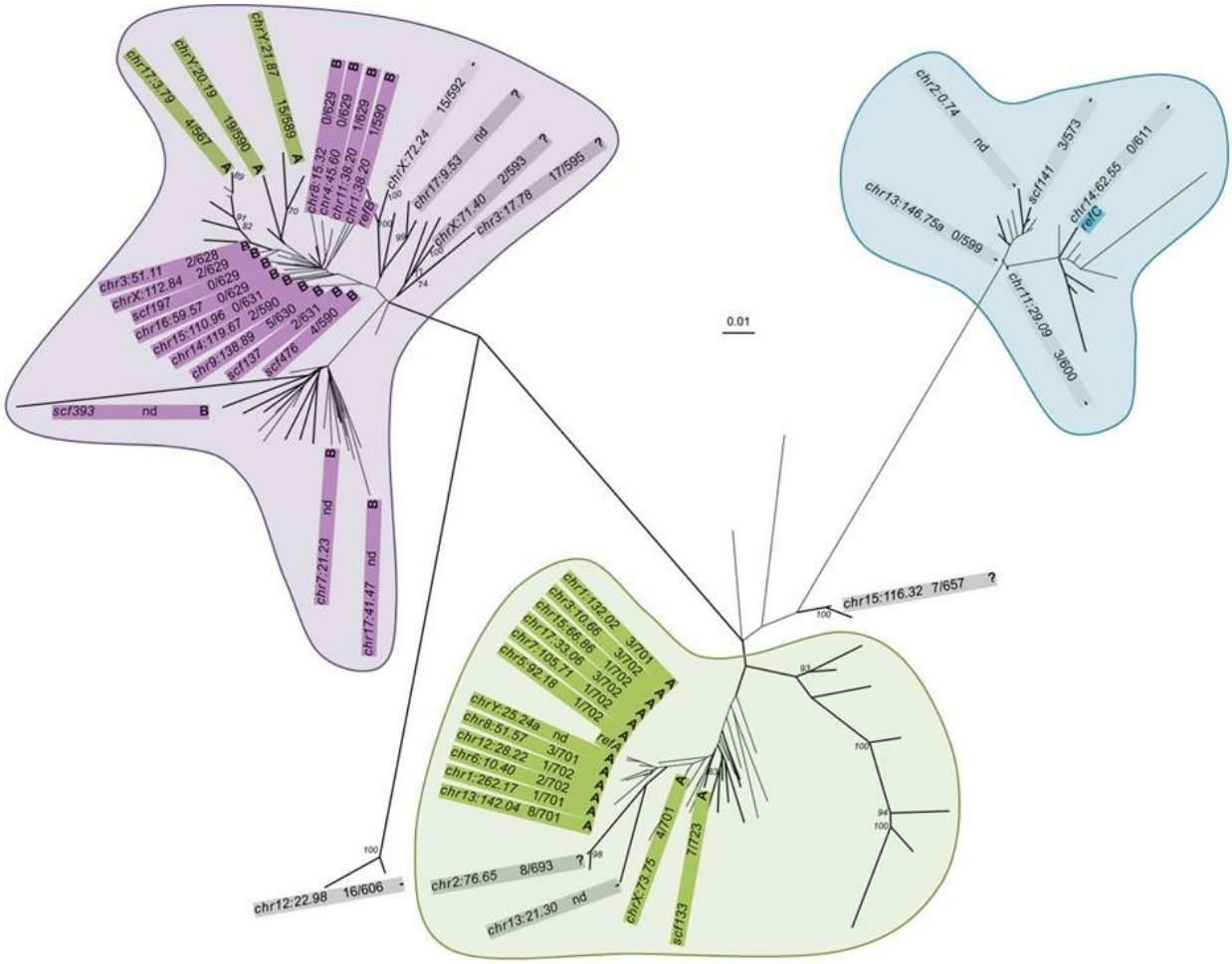

Figure 2 Phylogeny of PERV from the pig reference genome SusScrofa 11.1. LTR sequences were extracted from the genome and aligned. The tree is based on a maximum likelihood tree, with branches occurring also in most parsimony depicted in bold and branch nodes that occurred more often in 70 out of 100 genetic distance trees indicated. For better resolution, the solo-LTR are not indicated by name, but pairs of LTR that flank a provirus are given by their chromosomal position, the differences between the two LTR and the characterization of the sub-family based on the *env* genes. For better indication, proviruses with *env* of the PERV-A are marked in green, PERV-B is marked in magenta and PERV-C is marked in blue. If one end of the provirus has been affected by large genomic deletions, the difference of LTR cannot be determined ("nd"). If the sequence quality of the *env* gene was improper it is indicated by a "?" and if the *env* gene is affected by larger deletions the *env* gene is unknown ("-"). In both cases, the proviruses are marked in grey. (analysis done by N. Klymiuk)

In the second attempt, PERV-C proviruses were identified by Targeted Locus Amplification (TLA) sequencing (DE VREE et al., 2014) on a commercial basis by Cergentis (Cergentis, Utrecht, the Netherlands). The load of PERV-C was determined in four different individuals (pig number 1476, 3990, 4504 and 4686) from our breeding herd (**Figure 3**). In total, 11 proviruses of PERV-C have been identified and sequenced and specific PCRs for each integration site have been established by I.Kola during the preparation of her doctoral thesis "Deleting PERV-C infectious potential of donor pigs for xenotransplantation".

CERGENTIS
COMPLETE GENE SEQUENCING

Chr:Mb	1476				3990				4504				4686				remarks	Exact integration site
peak	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		Integration or genome position
Chr1:3	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr1:3014790-3014787
Chr1:20	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	?? Follow up.
Chr1:41	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr1:41140525-41140524
Chr2:77	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	Good peak, PERV in genome	
Chr2:100	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Odd shaped peak	
Chr2:110	-	-	-	-	-	-	-	-	+	+	?	?	-	-	-	-	Good but low coverage peak	Chr2:109094929-109094926
Chr3:40	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Non-specific	
Chr3:142	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Repeats	
Chr4:135	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	
Chr5:23	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	Chr5:22743215-22743212
Chr6:4.7	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	Low coverage peak	
Chr6:41	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome, odd shaped due to incorrect genome assembly?	Chr6:40976751-40976748
Chr7:18	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	Good peak, no PERV in genome	Chr7:18356720-18356717
Chr7:44	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr7:58	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr8:2	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr13:104	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	Good peak, no PERV in genome	Chr13:104501280-104501278
Chr14:31	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	Low coverage peak	
Chr16:79	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	Good peak, no PERV in genome	Chr16:78812732-78812729
ChrX:14	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	Good peak, no PERV in genome	

Figure 3 PERV-C identified in 4 genetically modified pigs from LMU, data provided by Cergentis, Utrecht, the Netherlands.

Based on the striking difference of PERV-C abundance in the reference genome versus four of our multi-modified piglets, the phylogenetic relationship between any LTR clustering with the PERV-C reference AF038600 was determined (**Figure 4**). Based on phylogenetic trees there is a clear separation of PERV-C LTR from the references of A, B. Although all PERV-C sequences appear highly homologous, there is a further clustering of the almost identical reference sequence, all PERV-C identified by TLA sequencing and the provirus remnant at

chr14:62.55Mb. The other LTR form a more diverse cluster, characterized by an ambiguous pattern of polymorphic sites and a significant number of unique positions. This interpretation of a highly homologous sub-cluster and a more diverse group is also supported by principle-component analysis.

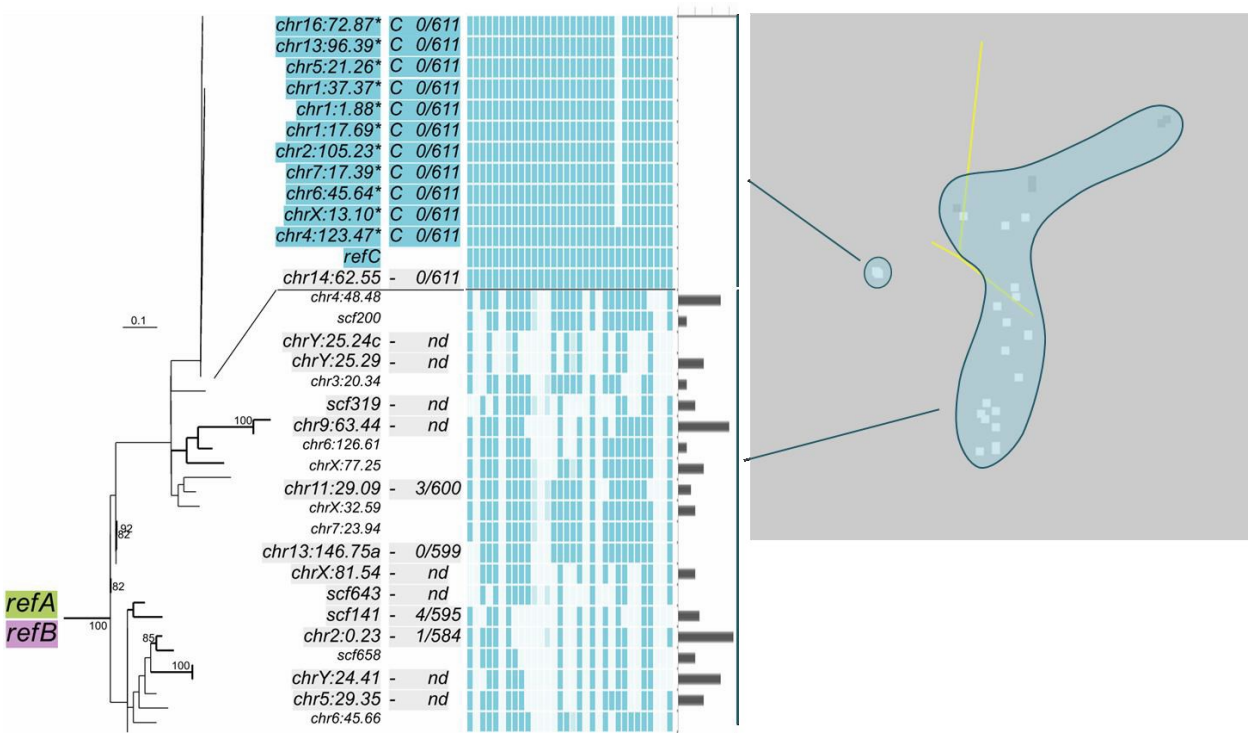

Figure 4 Analysis of LTR clustering with the reference PERV-C AF038600. A phylogenetic tree was generated according to the procedure described in Fig. 2 and rooted with LTR from the reference viruses of PERV-A and B (left panel). Designation of the LTR was done according their integration in the reference genome. Proviral sequences were designated as "C", if the env was corresponding to the reference virus (blue) and "-" if the env was largely lacking (grey). If both LTR of a provirus were entirely abundant, the difference between them is indicated versus their length. A position-wise comparison of the polymorphic sites in the alignment of all LTR is given in the middle panel. All positions identical to the reference virus are in blue whereas deviations occurring in more than a single sequence are given in pale. Differences that occur only in a single sequence are removed from the alignment and the numbers of such unique positions are given for each sequence in columns. Finally, principle component analysis of the alignment was performed by Jalview (right panel) and the clusters identified are correlated to the other two methods. (analysis done by N.Klymiuk)

The finding of two different clusters within PERV-C LTR was not really surprising, when one considers, that on one hand a group of proviruses lacking large parts of the env C has been found in the reference genome, whereas another group of largely intact proviruses has been identified by TLA sequencing. Surprisingly, however, one of the disrupted proviruses, chr14:62.55Mb strongly groups with the intact PERV-C whereas the other cluster showed a much higher diversity. It is important to consider that this higher divergence did not clearly support the assumption of an older age of this cluster, because differences between up- and down-stream LTR are relatively low. Still, based on this data set, one might come to the conclusion that the full PERV-C have appeared in the genome more recently, and it appears that they have come in at once, whereas the truncated PERV-C have populated the porcine genome during a longer period of time. Considering the impotence of these proviruses to spread in the genome on their own competence, the main question was then, by which way these proviruses have integrated in the pig genome. As proviral integration of gamma retroviruses results in a typical duplication of 4bp at the integration site due to integrase activity, I clarified the flanking sequences of the proviruses chr.14:62.55, chr.11:29.09, chr.2:0.23 and scf141. Consistently, all four integration sites revealed identical 4bp sections up- and downstream of the provirus, but the 4bp sections differed between each provirus (**Figure 5**). This finding matched the analysis of the 11 intact PERV-C, which has been performed previously by I.Kola (**Figure 6**). Other PERV-C proviruses either lacked one of their terminal ends, or were embedded in highly repetitive genomic regions, which prevented the independent amplification of the proviral ends. Taken together, this analysis shows that the truncated PERV-C are rather the product of integrase-mediated colonization of the genome, than a product of transposon-mediated accumulation or the product of pseudogene-like reverse transcription.

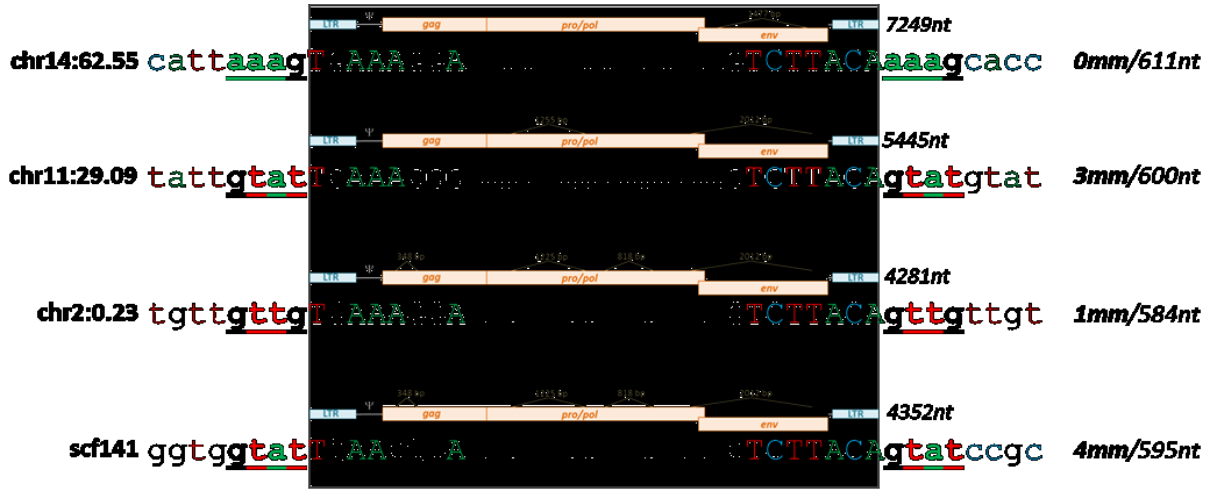

Figure 5 Flanking regions of the truncated PERV-C-like proviruses. The transitions from the provirus (capital letters) into the adjacent genomic region (small letters) is indicated for the proviruses at chr14:62.55, chr11:29.09, chr2:0.23 and scf141. The intimately adjacent positions are underlined and depicted in bold. The structure for each provirus is given schematically, with the positions and lengths of larger deletions indicated. At the very right, mismatches between LTR are shown.

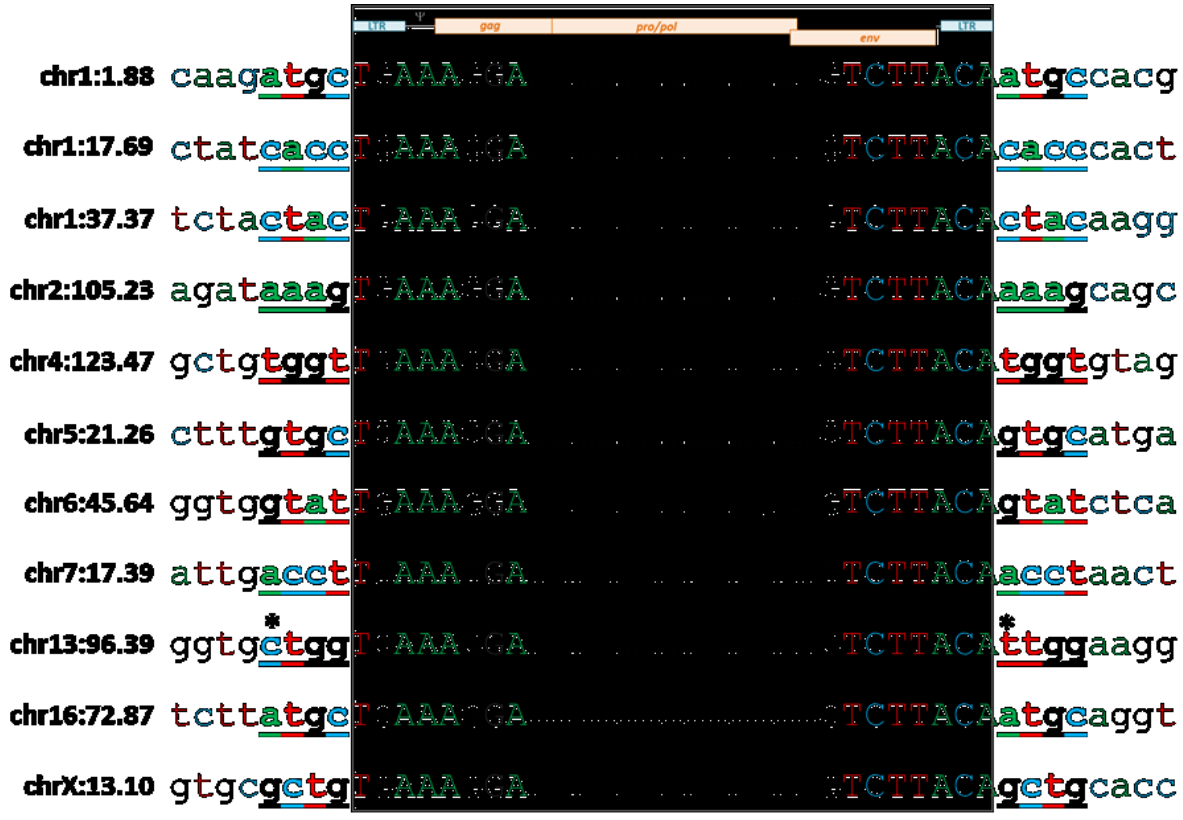

Figure 6 Flanking regions and 4 bp duplications of the 11 PERV-C proviruses identified by I.Kola.

As both, the intact PERV-C, as well as the disrupted proviruses appear to be the product of retroviral integration, we followed their pattern of inheritance throughout the history of our xenotransplantation donor herd. Each of a total of 148 animals was examined for the abundance of 11 PERV-C proviruses and 3 proviral remnants (**Table 13** and **Table 14**). Basically, it appeared that none of the animals had all of the proviruses in their genome and none of the proviruses appeared in all of the animals. In more detail, it became evident that any proviral integration was inherited to the offspring in a strictly Mendelian pattern throughout 8 generations of breeding (**Figure 7**). Following the stochastical principles behind inheritance and the relatively strong inbreeding within our donor herd, it was not surprising that some of the proviruses were lost by time, whereas others increased in their frequency within the herd as long as no attention was paid to the PERV-C-population, when animals were selected for breeding. After identification of the integration sites, breeding animals were selected for their PERV-C load, and a slow decrease of PERV-C in the herd has been achieved in the meanwhile.

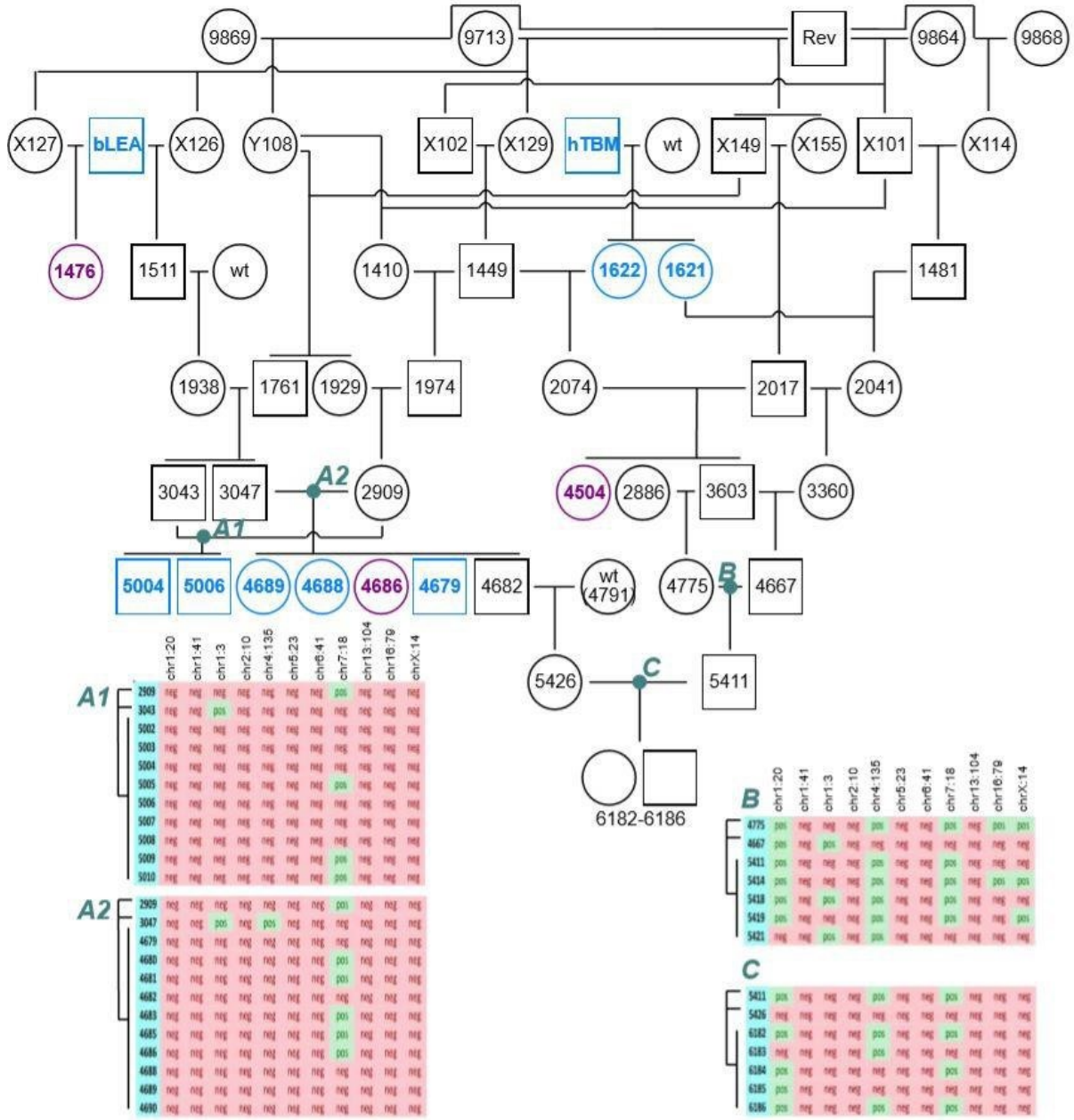

Figure 7 Inheritance of proviral sites in the xenotransplantation donor herd. The breeding history started by mating a cloned GTKO.hCD46 boar (Rev) with HLAE-transgenic sows. Later on, boars being transgenic for hTBM or INS-LEA (bLEA) were introduced into the mating scheme. For the ease of understanding, the genetic constellation of the animals is not indicated. Circles symbolize sows and boxes represent boars. Individuals that have been used for TLA-sequencing are depicted in magenta; individuals that are entirely free of PERV-C env are highlighted in light blue. The inheritance pattern of proviruses at specific nodes and for selected integration sites (marked by capital letters) is shown in separate tables.

5 Discussion

My working on the maintenance and sanitation of the LMU´s breeding herds for xenotransplantation, the veterinary management of the animals, as well as the providing of animals, tissue, or blood samples to different research projects allowed the contribution to distinct studies and facilitated insight into different aspects of xenotransplantation, ranging from hygiene aspects to preclinical research and molecular details of antibody-mediated complement activation.

5.1 Perspectives of xenotransplantation in comparison to alternatives

As a result of the enormous relevance of life-threatening heart diseases, in particular in the Western World, tremendous efforts are being undertaken in providing treatments to human patients. Evidently, many more attempts than xenotransplantation are being considered, and some of them are clinically used. Maybe the most widely used are left (or biventricular) assist devices (KIRKLIN, 2014; PATEL et al., 2014; KIRKLIN et al., 2015), which have been initially inserted as a bridge-to-transplant support, but the devices are increasingly used, also as destination therapy, implying that no allotransplant will be carried out (reviewed in MOU et al., 2015). An unfortunate complication of mechanical assist devices is very often sensitization to human leucocyte antigens (HLA) antigens, as a consequence of the multiple blood transfusions needed during the process. This normally exacerbates the search for a fitting deceased donor heart and compromises the value of mechanical assist devices as bridging therapy (COOPER & TEUTEBERG, 2010). The total artificial heart (TAH) is another, or, in many cases, the last option for those patients, who cannot be rescued by assist devices alone (COPELAND et al., 2012) or when allografts fail (QUADER et al., 2013). However, TAHs have their limitations due to limited durability (in average less than 5 years) (reviewed in GOERLICH et al., 2016) and impairment of their users' well-being and comfort with their sizeable external parts and noisy, large pump (SUNAGAWA et al., 2016).

A popular approach for treating many diseases is the application of pluripotent stem cells (PSCs) (CARPENTER et al., 2009; FOX et al., 2014). Basically, there are three categories of stem cells: first, there are permanent stem cells in most mature tissues with high turn-over rates,

which are responsible of taking care of tissue preservation. Then embryonic stem cells from culture of preimplantation embryos, which are seen as pluripotent, as they can differentiate in any cells existing in the human body. And lastly reprogrammed adult cells, referred to as induced PSCs (iPSCs) (reviewed in LANE et al., 2014). The PSCs can be obtained by manipulating the stem cell microenvironment, or "niche", to facilitate repair by endogenous stem cells (reviewed in LANE et al., 2014). The niche was first described by R. Schofield in 1978 (SCHOFIELD, 1978) as hypothetical interaction of stem cells and their environmental cells. There are niches in many tissues, for example in skin (FUCHS, 2009), intestines (BARKER, 2014; TAN & BARKER, 2014), and the nervous system (CONOVER & NOTTI, 2008). Regarding the heart or the myocardial tissue, it was shown, that PSCs can differentiate into cardiomyocytes in animal models (LAFLAMME et al., 2007; XIONG et al., 2013). Improvement of the function of infarcted hearts, that were transplanted with mouse and guinea pig PSC-derived cardiac progenitors (SHIBA et al., 2012) and even long-term benefit has been reported in the repair of a damaged heart (HARRIS et al., 2007; SCHOLL et al., 2010). Importantly, induced PSCs proved as an innovative and attractive source of PSCs, simply generated from differentiated cells by somatic cell reprogramming (TAKAHASHI & YAMANAKA, 2006). Still, in many cases organs are so severely damaged that cellular regeneration is believed to be of little use and replacement by a "new" heart is the only chance to save patients´ lives (reviewed in MOU et al., 2015).

On the other hand, the availability of personalized, autologous iPSC, stimulated also the field of tissue engineering (reviewed in BERTHIAUME et al., 2011). The term tissue engineering comprises living cells, biocompatible materials and suitable biochemical factors for creating tissue-like structures. State-of-the-art approaches use scaffolds made from naturally derived and synthetic polymers, bioresorbable inorganic materials, hybrids, or decellularized tissue scaffolds (reviewed in PINA et al., 2019), which are then cellularized either with iPSCs or donor cells. This was done, for example with rat kidneys and discarded human transplant kidneys, using human inducible pluripotent stem cell–derived endothelial cells for re-endothelialization (LEUNING et al., 2019). This may be a possible application in the future, but, as the heart is a very complex organ and consisting of multiple cell types, with various functions, an appropriate multi-cellular composition, which resembles the heart does not exist until now (OWEN & HARDING, 2019).

Another development in the growing field of tissue regeneration is blastocyst complementation (CHEN et al., 1993). The generation of a whole functioning human organ,

solely derived by the patient's iPSCs is the ultimate goal, as it would significantly reduce the immune response (reviewed in MOU et al., 2015; reviewed in OLDANI et al., 2017). The basic idea is that organs are formed, by cells from a complementary individual, if a developmental niche is provided by the host. Concept was proven when mouse iPSC-derived kidneys were generated in mice (USUI et al., 2012). In a further study, cross-species chimeras were generated, when rat-derived iPSCs formed the pancreas in mice (KOBAYASHI et al., 2010). Later it was shown, that the blastocyst complementation technique can be applied to the pig (MATSUNARI et al., 2013). Further, human-pig chimeras were reported at embryonic stage (WU & IZPISUA BELMONTE, 2015; WU et al., 2017), but the experiments were not carried out until birth. Thus, the viability of such individuals remains elusive and the practicable terms of this approach are unclear. Very importantly, the concept of blastocyst complementation has also raised significant ethical concerns (HERMERÉN, 2015; SHAW et al., 2015).

The latest innovation in the expanding field of bioengineering is 3D tissue printing (reviewed in MURPHY & ATALA, 2014). As the used technologies for 3D printing were originally not intended for the use with biological materials, the first printers used for bioprinting were modified, commercially available, ink-based printers (XU et al., 2008) and the selection of the materials is one major task. Not only do they have to withstand the printing process, but they should supply the wanted characteristics for the tissue to be composed. Only recently a proof-of-concept study was performed where contractile cardiac tissue constructs were printed, which were able to perform synchronal contractions (WANG et al., 2018). Additionally, two very promising studies printed human "hearts". One printed small-scale human hearts, as a proof-of-concept, that anatomical, volumetric and complex structures can be printed, using a personalized hydrogel as matrix (NOOR et al., 2019) and the other group was able to directly print collagen and thus bioengineer tissue parts of the human heart at various sizes and even a neonatal-sized human heart (LEE et al., 2019). Though these are very encouraging results, the 3D bioprinting technology still needs to overcome significant obstacles, like the generation of countless millions of cells needed to print large tissues or the creation of a feasible workflow for clinical adaption and still the most challenging, is the creation of a fully functional 3D bio printed organ.

In the context of many promising, but not yet clinically applicable approaches, the recent publication of consistent long-term survival of xenotransplanted pig hearts in primates for up to 6 months resembles a major breakthrough (LANGIN et al., 2018). Not only would pig

breeding facilitate the regular, reliable, and continuous production of donor individuals, but also allow a much better planning of transplantation events and the application of techniques, that are well established in the course of allotransplantation. Importantly, the physiological function of a pig heart is apparently life-supporting in a living primate, a fact that has not been yet proven for any of the above described biological replacements. Some aspects such as the controlling of the pig heart´s intrinsic growth potential need definitely further consideration, but the induction of a genetically induced dwarfism in the donor pigs (HINRICHS et al., 2018) might be a sufficient approach to tailor the size of the donor pigs. Similarly, the so far significant immunosuppressive burden might be decreased, when local T-cell control is induced, such as by the expression of PDL1 (BUERMANN et al., 2018) or by the secretion of LEA29Y (BAHR et al., 2016). The most critical point of supplying donor animals for xenotransplantation, however, is the threat of transmitting pathogens from the donor to the recipient, and, if becoming pandemic, among the human population. Therefore, a strict hygiene standard of the donor pig is necessary and the exemplified sanitation of the hygiene status in a donor herd was the core of my thesis.

5.2 The way towards an approved barrier facility for clinical trials

It is important to mention, that so far, no specific regulations exist that describe the maintenance of donor pigs for xenotransplantation, rather there are only recommendations or guides, most notably in the context of clinical trials. Most of the guidelines published by WHO, FDA or EMA deal with more general considerations, such as standards for facility construction or the importance of keeping notes about feeding, water supply, deposition of waste and so on. As such, the “Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans” (FDA, 2003) claims, for example, that barrier facilities should be built, accredited by the AAALAC and run in accordance with the “Guide for the Care and Use of Laboratory Animals” from the National Research Council (NRC, 2011). Being claimed as the “Gold Standard” of laboratory animal care (SPIZZO et al., 2016) the “Guide for the Care and Use of Laboratory Animals”, is, however, rather written for specialized mouse or small rodent barrier facilities, so that only the basic principles defining documentation, training of employees, veterinary care, etc. are helpful. On the other hand, the “Guide for the Care and Use of Agricultural Animals in Research and Teaching” by the Federation of Animal Science Societies (FASS) (FASS, January 2010) gives

more specific advice for keeping pigs as an agricultural animal. But the guide has been mainly written for the use of large animals in e.g. field research for agricultural purposes or teaching. Although it has been updated, only little information is given on the specific demands in biomedical research, and therefore the described standards of biosecurity, SOPs, veterinary practice and GMP standards are helpful for general maintenance principles, but they do clearly not fully cover the demands of donor pigs for xenotransplantation.

One obviously critical point of housing donor pigs for xenotransplantation will be the regulation of access to the pigs. It is well known, that the number of individuals entering the facility and the frequency of entry must be minimized. At least a complete documentation of external people visiting the facility should be kept. Therefore, we installed a sort of "Guestbook" at the entry of CiMM where every guest, external technician and member of any other workgroup have to declare, that he/she had no contact to pigs for at least 2 days and that he/she is not ill at the moment or was not ill in the past days. Furthermore, he/she declares his/her agreement with the use of the double door changing system, including the use of the shower and the change of clothes included in this system. A further recommendation from the "Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans" by the Food and Drug Administration (FDA, 2003) proposes to even limit the traffic of caretakers between distinct compartments of the facility or between different groups of animals. Working with more than one herd on a single day should only be done if validated SOPs for disinfection and decontamination are employed. This regulation, however, cannot be executed at CiMM at the present timepoint, because this is only possible at very high personal and monetary expense.

Surprisingly, European regulations, so far, consider only the production of cellular xenotransplantation products in the clinical xenotransplantation context, the so-called Advanced Therapy Medicinal Product (ATMP), and in detail describe requirements for isolation, treatment and cultivation of those products, based on the EU Regulation 1394/2007 (PARLIAMENT, 2007) (reviewed in SCHUURMAN, 2015). Unfortunately, this regulation is, coincidentally with any other regulations, not very informative on the requirements under which donor animals should to be kept. In very common and general terms regulatory authorities like WHO (WHO, 1998), EMA (EMA, January 1, 2010) or FDA (FDA, 2003) claim, that the test regimen should be tailored to the purpose of the animals' use, the test regimen should be established in accordance with experts from the fields of veterinary medicine,

virology and epidemiology and thus, the established regimen should be reviewed at given timepoints to keep it up with the advance of scientific knowledge. It is, however, evident that there are a lot of different opinions on these scientific considerations, and there is little to no consensus of an adequate and feasible hygiene monitoring. With a certain reputation, the International Xenotransplantation Association (IXA) has made recommendations which are extremely extensive, covering more or less any pathogen that has been associated with pig at any time (SCHUURMAN, 2009). Meanwhile, their current version "First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes--Chapter 2a: source pigs--preventing xenozoonoses." (SPIZZO et al., 2016) condensed this initially proposed test regimen to a more practicable extent. Other authors aimed at the same purpose and published their personal suggestion of test regimen (GARKAVENKO et al., 2004a; GARKAVENKO et al., 2008a; WYNYARD et al., 2014; FISHMAN, 2018). Regarding bacteria and viruses there is certain coincidence between these studies and the updated IXA consensus statement, whereas the agreement is less pronounced regarding fungi or protozoa (**Table 11**).

	Fishman 2018	**Spizzio 2016 (IXA)**	**Wynyard 2014 New Zealand**	**Garkavenko 2004+2008**	**CiMM**
Bacteria					
Leptospira Serovar Tarrasovi	✓	✓	✓		
Leptospira Serovar Hardjo	✓	✓	✓		
Leptospira Serovar Pomona	✓	✓	✓		
Leptospira interrogans					✓
Mycoplasma hyopneumoniae	✓	✓	✓		✓
Campylobacter	✓	✓	✓		
Yersinia	✓	✓	✓		
E.coli K88		✓	✓		
Salmonella spp.	✓	✓	✓		✓
Mycobacterium tuberculosis	✓				
Shigella	✓				
nontuberculous mycobacteria	✓				

+ M.bovis					
Listeria monocytogenes	✓				
Brachyspira hyodysenteriae /pilosicoli					✓
Lawsonia intracellularis					✓
Pasteurella multocida					✓
Actinobacillus pleuropneumoniae					✓
C. perfringens					✓
Haemophilus parasuis					✓
bact. pool examination from feces					✓
Brucella suis	✓				
Viruses					
MRV			✓		
HERV-K			✓		
PCV2	✓	✓	✓	✓	
PCV1	✓	✓	✓		
PLHV	✓			✓	
PLHV2		✓	✓		
PCMV	✓	✓	✓	✓	✓
Rotavirus A-C		✓	✓		✓
Reovirus		✓	✓		
PTV		✓	✓		
PEVB		✓	✓		
PHEV		✓	✓		
HEV	✓	✓	✓		✓
BVD		✓	✓		
SuHV-1 (AujD)	✓	✓	✓		
PPV	✓	✓	✓		
PRRSV	✓	✓	✓		✓
EMCV	✓	✓	✓		
PERV		✓	✓	✓	✓
Adenovirus	✓				
Rabies virus	✓				
TGEV					✓
Influenza virus (human)	✓				
Influenza virus (swine)	✓				✓

Protozoa/ Parasites					
Toxoplasma gondii	✓	✓	✓		
Ascaris suum	✓				
Cryptosporidium/ Microsporidium spp.	✓	✓	✓		
Echinococcus spp.	✓				
Giardia spp.	✓				
Isospora sp.	✓	✓	✓		
Strongyloides sp.	✓				
Trichinella spiralis	✓				
endoparasites (flotation and sedimentation)					✓
Trypanosoma spp.	✓				
Fungi					
Aspergillus sp.	✓				
Candida sp.	✓				
Cryptococcus neoformans	✓				
Histoplasma capsulatum	✓				

Table 11 Proposed test regimens adapted from different authors (GARKAVENKO et al., 2004a; GARKAVENKO et al., 2008a; WYNYARD et al., 2014; FISHMAN, 2018) and IXA (SPIZZO et al., 2016) compared to the test regimen of CiMM. (refer to **Table 1**)

(MRV: mammalian orthoreovirus, HERV-K: Human endogenous retrovirus K, PCV1 /2: porcine circovirus 1/2, PLHV: porcine lymphotropic herpesvirus, PLHV2: porcine lymphotropic herpesvirus 2, PCMV: porcine cytomegalovirus, PTV: porcine teschovirus, PEVB: porcine enterovirus, PHEV: porcine hemagglutinating encephalomyelitis virus, HEV: hepatitis E virus, BVD: bovine virus diarrhea, SuHV-1 /AujD: suid alphaherpesvirus 1/ Aujezsky's disease, PPV: porcine parvovirus, PRRSV: porcine reproductive and respiratory syndrome virus, EMCV: encephalomyocarditis virus, PERV: porcine endogenous retrovirus, TGEV: Transmissible gastroenteritis coronavirus)

As explained in our study on hygiene sanitation during the population of CiMM, we did not only screen for PCMV but also for a various number of other pathogens, listed exemplary in **Table 12** (EGERER et al., 2018). Within the study, the 91 F1 piglets, and consequently all gilts which became ET recipients, were monitored very tightly over a period of 14 months. This extent has not been achieved in the F2 generation, i.e. the first gm offspring, due to several reasons. First, the sequential farrowing of ET recipients logistically complicated the sampling of each animal at the same age and prevented the testing of all animals at the same timepoint (**Figure 8**). Second, with increasing numbers of pigs in CiMM, individual testing becomes very cost-intensive and, third, the establishment of breeding herds out of only a few founding animals is a critical undertaking. Loss of animals needs to be avoided under any circumstance, which is further complicated when certain pig lines are stress sensitive such as the Duchenne muscular dystrophy (DMD) pig model (KLYMIUK et al., 2013) or Laron animals (HINRICHS et al., 2018). Thus, only partial blood sampling has been carried out on representative animals. Still, all of the 19 F2 animals tested in the study have been proven free of the tested pathogens. In the meanwhile, and during the continuing settlement of the different breeding lines, the test regimen is being continued according to the publication. The reduced frequency and scale of sampling is, importantly, not only dictated by the available resources at CiMM, but also according to the intentions of animal welfare: while the tight monitoring of animals during the population of CiMM was necessary to prove the effect of the sanitation, it would meanwhile resemble a significant burden for the animals without essential gain of knowledge. This condition might change, once donor animals for clinical xenotransplantation will be delivered, but at the moment the required hygiene standard has not been finally clarified. Furthermore, and according to the FDA “Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans” (FDA, 2003) it might become obligatory to maintain donor animals separated from any other pigs that are not being dedicated for this purpose. Thus, at the present timepoint, maintenance and surveillance of a common hygiene standard for experimental animals under the “FELASA recommendations for the health monitoring of breeding colonies and experimental units of cats, dogs and pigs” (REHBINDER et al., 1998), is the opportune way to go, both, from the economic point of view as well as from the standpoint of animal welfare.

TEST	SAMPLE AMOUNT	POSITIV	NEGATIV	QUESTIONABLE	STATUS
bacteriological examination	45	0	45	0	finished
APP-AK	45	0	45	0	finished
C.perfringens-AK	45	0	45	0	finished
HEV-AK	45	0	45	0	finished
Heamophilus parasuis-AK	45	0	45	0	finished
Lawsonia intracellularis (PIA/Ileitis) - AK	45	29	16	0	finished
Mycoplasma hyopneumoniae -AK	45	0	45	0	finished
Pasteurella multocida (toxA) - AK	45	0	45	0	finished
PRRSV - AK	45	0	45	0	finished
Influenza - AK	45	0	45	0	finished
TGEV-AK	45	0	45	0	finished
Leptospira-AK (MAT)	45	0	45	0	finished
Brachyspira - PCR	15	0	15	0	finished
Lawsonia - PCR	15	0	15	0	finished
Mycoplasma hyopneumoniae - PCR	45	0	45	0	finished
Pasteurella multocida (toxA) – PCR	15	0	15	0	finished
Influenza A - PCR	45	0	45	0	finished
Salmonella (culture)	15	0	15	0	finished

Table 12 Exemplary screening results table, adapted from the ones we receive from Vaxxinova. (Vaxxinova GmbH, Münster, Germany)

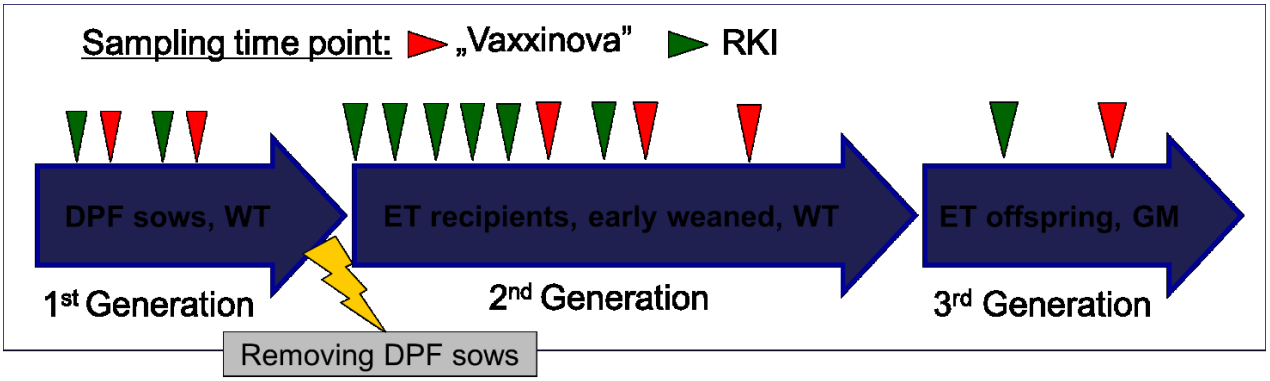

Figure 8 Showing coarsely the different sampling time-points for Vaxxinova and the Robert-Koch-Institute (RKI) of the three different pig generations, populating CiMM to establish a PCMV free pig herd.

5.3 Novel pathogens to be considered for donor herd safety

Eventually, the definition of a test regimen will depend on the responsible authority, which in Germany is the Paul-Ehrlich-Institut (PEI). Evidently, any initial test regimen definition will be based on the expert opinions in the field, but evidently as well, the requirements for testing animals will be refined after initial definition, according to the Fishman statement "It seems likely that *the way in which the level of safety is achieved need not be uniform so long as the transplanted tissues do not pose a microbiologic hazard to the recipient*"(FISHMAN, 2018). The recent discovery of Porcine Circovirus 3 (PCV3), a new possible pathogen in our previous breeding herd at LVG (KRUGER et al., 2019) might serve as a good example for developing standards in hygiene monitoring. PCV3 was first described in 2016 in pigs with cardiac and multi-systemic inflammation (PHAN et al., 2017), in mummified fetuses aborted from sows with porcine dermatitis and nephropathy syndrome (PDNS)-like lesions and in sows that died acutely with PDNS-like clinical signs (PALINSKI et al., 2017). However, PCV3 is not a new virus, but has been found in retrospective studies on samples dating back to 1993 in Sweden (YE et al., 2018) and to 1996 in Spain (KLAUMANN et al., 2018) and China (SUN et al., 2018). Although so far, the relevance of PCV3 for xenotransplantation remains elusive, its recent appearance in pig populations illustrates the dynamics of pathogen evolution. It is their very nature that viruses in general, and circoviruses in particular, are genetically diverse and can infect a wide range of hosts, with documented cross-species transmission (LI et al., 2010; LI et al., 2011). For better understanding the forces behind the latter, the idea of a "host-agent-environment triangle" has been developed (reviewed in DAVIES, 2012). This is a conceptual model that takes into account the interactions between environment, host and an infectious (or abiotic) agent (SCHOLTHOF, 2006). This model can be used to predict epidemiological outcomes in plant health and public health and was first established by George McNew in the 1960s (MCNEW, 1960). According to McNew, six interacting factors determine the development of an economically important disease: the climate of the physical environment (for example drought or humidity), the duration of the infection period, prevalence of the pathogen, virulence of the pathogen, the age or maturity of the host and its particular susceptibility to disease. By reduction to these parameters a host-agent-environment triangle can be used as a predictor of new or variant pathogens, spreading in a dense population, which definitely reflects the present conditions of agricultural pig housing. In addition, in the growing agricultural sector, genetic selection of the potential host species has led to almost genetic

uniformity (EDFORS-LILJA et al., 1998), which must also be considered as essential factor for disease resistance and the development of newly emerging diseases. Livestock production in large herds represents an excellent environment to alleviate the transmission and expansion of viruses, contributing to the pathogen evolution through mutation, recombination and reassortment (NICHOL et al., 2000; LA ROSA et al., 2012; CORREA-FIZ et al., 2018). The continuing intensification of agricultural production might have been a major driver for establishing PCV3 in the pig, and the extensive exchange of animals, human beings being in contact with the animals, and material from agricultural facilities might have supported the spreading of PCV3 in pig population all over the planet. Therefore, raising large livestock herds without implementing enhanced biosecurity may lead to populations particularly vulnerable to disease emergence (PULLIAM JULIET et al., 2012) (reviewed in DAVIES, 2012). Emerging disease events, particularly in Asia, underline the importance of biosecurity measurements, as it still remains a hot-spot for novel zoonotic diseases, arising from inter-species contact, for example new influenza strains (WEBSTER, 2002), Nipah virus (PULLIAM JULIET et al., 2012) or SARS (LI et al., 2005). In contrast to the situation in Asia, where inter-species contact is common and enables the development of zoonotic diseases, the trend in developed countries has led to increased herd sizes with only one species kept, condensed at one place. As a consequence of dense livestock populations, pathogens often show high virulence in individual species, but due to the diminished inter-species contact, pathogens appear relatively host specific (reviewed in DAVIES, 2012). However, recent findings show that this is not true in all cases. For example, porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) have been shown to infect human cells (HATTERMANN et al., 2004; LIU et al., 2019) and thus circoviruses may pose a risk on a xenotransplant recipient (reviewed in DENNER & MANKERTZ, 2017). For PCV3 the picture is less clear. Evidently, the virus was also found in wild boars (FRANZO et al., 2018a; KLAUMANN et al., 2019; PRINZ et al., 2019) and it is suggested that wild boars may be a potential reservoir for PCV3 infection. To fill the missing epidemiological data, plenty of studies were carried out to expand the knowledge on PCV3 evolution, transmission, spreading patterns and impact on pig health. It was possible to detect PCV3 by PCR in nasal swabs (FRANZO et al., 2018c), oral fluids (KWON et al., 2017), feces (COLLINS et al., 2017; KLAUMANN et al., 2019), semen (KU et al., 2017), in sow colostrum (KEDKOVID et al., 2018) and even in sponges collected from trucks after sanitation (FRANZO et al., 2018b). Further, PCV3 was detected in dog sera in China (ZHANG et al., 2018) and in all

organs of the recipient baboons in the pig-to-primate xenotransplantation model (KRUGER et al., 2019), which suggests trans-species transmission. But up to now, there is no other (than these two) evidence that PCV3 is capable of infecting other species. It can be only suggested, that there was replication of PCV3 in the baboons, since the virus was found in all organs from the four baboons and the virus load increased with the survival time (KRUGER et al., 2019). As "transmission" describes the way or circumstances a pathogen needs to get from its current host to the next healthy individual and there are five big categories of means of transmission: contact, food- or water-borne, air-borne, vector-borne and perinatal (NELSON et al., 2001) and PCV3 was further detected on so many different body fluids and even sponges, it can hardly be guessed which transmission routes PCV3 takes.

Regarding xenotransplantation, transmission, infection and disease potential of PCV3 remains totally unclear at the moment. Experiments to infect human cell line 293 were not successful in our study (KRUGER et al., 2019), but does evidently not prove that PCV3 is not capable of infecting any human cells at all. Further investigations have to be done to understand the possible risk that PCV3 may pose on xenograft recipients, but at the moment it appears that testing for PCV3 in our breeding herd might be senseful, but not ultimately necessary.

5.4 Heredity of PERV-C proviruses in our xenotransplantation donor herd

Due to their endogenous nature, PERVs resemble a special aspect of xenotransplantation safety. Their integral constitution in the genome complicates their removal. A very important aspect is also that the threat that PERVs are representing is not fully clear. Evidently, the infection assays that have been documented decades ago (ARMSTRONG et al., 1971; LE TISSIER et al., 1997; PATIENCE et al., 1997; TAKEUCHI et al., 1998; SCOBIE & TAKEUCHI, 2009), were so far not confirmed by *in vivo* experiments. Evaluation of clinical xenotransplantation studies using capsulated islet grafts did not show evidence for virus transmission to patients (GARKAVENKO et al., 2004b; WYNYARD et al., 2014; MOROZOV et al., 2017; reviewed in DENNER, 2018).In addition, there is no animal model displaying the *in vivo* trans-species transmission situation of PERVs, and until now, no assays for monitoring and for the detection of PERV transmission *in vivo* have been certified. However, it is clearly recommended to use PERV-C free animals as donors for xenotransplantation, to avoid the possibility of recombinant PERV-A/C occurrence (DENNER et al., 2009). We therefore wanted to assess the amount of PERV-C in our herd of donor pigs for xenotransplantation. We could not identify any PERV-C

in the reference genome of the pig by scanning it with the *env* sequence, whereas PERV-C proviruses were found when examining the reference genome for the associated *LTR* sequences. The finding, that some of these LTR descend from truncated proviruses, raised the question how these truncated proviruses (the proviruses chr.14:62.55, chr.11:29.09, chr.2:0.23 and scf141) could have colonized the genome of the pig. A very important hallmark of endogenization of retrovirus is its integration into the genome of germ cells after which the retrovirus can endure as stable provirus for multiple generations (BOEKE & STOYE, 1997). A typical sign for retroviral integrase activity, amongst others, is a duplication of a certain number of host bp DNA (HISHINUMA et al., 1981), being characteristic for different viruses, for example, a 6 bp duplication for avian sarcoma/leukosis virus (ASLV), 4 bp duplication for MLV and 5 bp duplication for HIV-1 (reviewed in ANDRAKE & SKALKA, 2015). Although there are studies that suggest that solo-LTR, the most truncated form of a provirus, can amplify in the genome via classical retrotransposition (HEIDMANN et al., 1988; JUNGMANN & TÖNJES, 2008), we conclude from the consistent 4 bp duplication in their flanking region, that the truncated proviruses, similar to the full-length PERV-C, have entered the pig genome by retroviral integration. It is clear that a truncation of the *env* gene might have happened during reverse transcription, after a retroviral particle has entered a germ cell or that, even later, the truncation has occurred at the proviral stage. In both cases, such a provirus might then represent an evolutionary dead end, from the retroviral point of view, being incompetent for replication. The finding that all truncated PERV-C-like proviruses have an identical gap in the *env* gene, however, challenge this terminal faith hypothesis. As the provirus is truncated and lacks parts of its *env* gene, an amplification by retroviral re-infection depends on the generation of infectious particles by intact proviruses and the packaging of the truncated proviral transcript into such particles. From such particles new truncated proviruses might accumulate in germ cells after infection and reverse transcription. Although I did not find evidence in the literature for such a “hitchhiking” scenario, this is the most conclusive explanation for our finding of independent retroviral integration of truncated proviruses.

Another interesting aspect of our examination of PERV-C is the timepoint of their integration. Based on the molecular-clock hypothesis (reviewed in HO & DUCHENE, 2014) the age of a provirus can be estimated by the mutations in the two *LTR* sequences flanking the proviral genome, as they develop separately from each other after integration into the host’s genome (KLYMIUK et al., 2006). It has been estimated that PERVs persists in the pig genome for 7.6

million years, at most (TONJES & NIEBERT, 2003). The fully identical LTR pairs of the full length PERV-C and the maximum number of 4 mismatches between the LTR in truncated proviruses, however, suggests a much younger age of this specific subfamily. This is also consistent with the very diverse pattern of PERV-C integration sites in our breeding herd. Of note, none of the PERV-C proviruses were found in all animals. Even, if it is considered that our breeding herd is a mixture of different more or less "modern" agricultural breeds, such as German Landrace, German Large-White, Duroc, Piétrain, Schwäbisch-Hällisch, and others, and might have introduced the different proviruses, these breeds are quite young in evolutionary terms, very likely a few hundred years at most. Although, the domestication lineages of European pig breeds are difficult to follow and might have been mixed occasionally with wild-boar genomes (FRANTZ et al., 2019), it is likely that PERV-C proviruses have not only entered the genome of domestic pigs via domestication of wild-boars, but also via infection in agricultural pigs.

By following the different PERV-C provirus integration sites in the animals used for establishing and maintaining the breeding herd at MABB, we revealed that the different PERV-C provirus integration sites were inherited vertically from parents to offspring in a Mendelian manner (**Figure 7**), which is consistent to endogenous retroviruses in chicken and mouse lines (VOGT, 1997; PATIENCE et al., 2001). Together with the finding that none of the PERV-C proviruses is present in all animals, this is an extremely useful finding because, this would facilitate the outbreeding of PERV-C proviruses from our donor pigs for xenotransplantation. Although, this would be a really time-consuming approach, limited in its power by the low numbers of animals produced, and the difficulties to balance the desire for low PERV-C copy numbers with the required combination of genetic modifications when choosing future breeding animals. Still, the proven feasibility to get rid of PERV-C simply by breeding makes this approach practicable, whereas alternatives such as the excision of given PERV-C proviruses (see Doctoral thesis "Deleting PERV-C infectious potential of donor pigs for xenotransplantation" by Ingrid Kola) or the global inactivation of PERV by deleterious mutations within the provirus (YANG et al., 2015; NIU et al., 2017) resemble a tremendous effort. In the future perspectives of generating novel models for xenotransplantation from scratch, it is also relevant to have PERV-C free individuals identified, as such will form the basis of generating PERV-C-free genetically modified lines.

5.5 Anti-Gal antibodies – a vital heritage

Immunosurgery is a very sophisticated and effective way to isolate the inner cell mass (ICM) from blastocysts, by complement-mediated lysis of the antibody-coated trophectoderm (TE), but the choice of the antiserum is one of the most critical factors determining the isolation efficiency (KUROME et al., 2019). As the enzyme *GGTA1* is functional in most species, but not in Old World Monkeys, apes and men (GALILI et al., 1988b; GALILI, 1993), the usage of serum of GTKO pigs and therefore the anti-Gal antibodies contained in the serum of these pigs, are, in contrast to the previously used antisera in the field of immunosurgery, well defined to one specific epitope and applicable to all species that express αGal epitopes (KUROME et al., 2019). The serum from our GTKO pigs, collected for this study, proved as a reliable and effective source of anti-Gal antibodies to target the αGal epitopes on the surface of the blastocysts from the different (mammalian) species used in this study. Even the youngest GTKO pigs, with only 5 months, had enough natural anti-Gal antibodies in their serum to induce complement-mediated lysis, though the serum of a two-year-old pig, showed an even stronger activity, which indicates, that the number of antibodies circulating in the blood increases over the time. This is in line with the idea that individuals lacking the αGal epitopes start to produce anti-Gal antibodies at a very early stage in life, after immune stimulation by bacterial carbohydrate epitopes at the age of 6 weeks (DOR et al., 2004; FANG et al., 2012; GALILI, 2013).

Of note, the effect we took advantage of, namely that anti-Gal antibodies induce complement-mediated lysis resembled its very natural function, the destruction and neutralizing of viruses presenting αGal epitopes which was seen in murine C retrovirus (ROTHER et al., 1995), PERV (TAKEUCHI et al., 1996), pseudorabies virus (HAYASHI et al., 2004), HIV (NEIL et al., 2005) and some others. This leads to the suggestion, that Old World Monkeys, apes and men, who are the only ones that lack the αGal epitopes, but produce natural anti-Gal antibodies somehow took advantage of this situation. The present theory about this advantage is, that an evolutionary mechanism named “catastrophic-selection” (LEWIS, 1962), by which entire parental populations are replaced by very few mutated offspring, which manage to survive a natural disaster, for example extinction by viral epidemics, took place 20-30 million years ago (reviewed in GALILI, 2019). Here, the parental population of ancestral Old World Monkeys and apes were replaced by their GTKO offspring, who was capable of destroying the viruses circulating, and thus was protected from extinction. Later in evolutionary history, the same scenario was repeated when early ancestors of *Homo sapiens*, synthesizing N-5-glycolyl-

neuraminic acid (Neu5Gc), were replaced by offspring lacking Neu5Gc and instead now only synthesizing N-acetyl-5-neuraminic acid (Neu5Ac), because of inactivation of their *cytidine-monophosphate-N-acetyl-neuraminic acid hydroxylase* (*CMAH*) gene (SHAW & SCHAUER, 1989; CHOU et al., 1998; IRIE et al., 1998; MUCHMORE et al., 1998; VARKI, 2010; SPRINGER et al., 2014). The event of catastrophic-selection sets apart from Darwinian natural selection and from genetic drift, as they occur as gradual changes during many generations, as a result from continuous selection (reviewed in GALILI, 2019), whereas in catastrophic-selection, one mutation accidentally appearing in a very small population of offspring, leads to resistance in a selective event that eliminates all of the parental population, lacking the required mutation within a short period of time. The synthetization of αGal epitopes in nonprimate mammals, lemurs and New World Monkeys indicates, that ancestral Old World Monkeys and apes were also capable of synthesizing αGal epitopes in early periods after the split from New World Monkeys, which is estimated to have occurred about 30-43 million years ago (STEIPER et al., 2004; SCHRAGO et al., 2013), but they went extinct, as mentioned above, because of lethal virus infection, most likely by an airborne, highly virulent, enveloped virus. These enveloped viruses have phospholipids and glycoproteins which form together the envelope (GALILI et al., 1996). To synthesize the carbohydrate chain portion of the glycoproteins, the virus depends on the enzymes of the Golgi apparatus of the host (ROBBINS et al., 1977; KORNFELD & KORNFELD, 1985). The glycosyltransferases in the host's Golgi apparatus add carbohydrate units to the developing chain of glycoproteins as it moves through the compartments of the Golgi apparatus. *GGTA1* adds the terminal galactose, provided by uridine-diphosphate galactose (UDP-Gal), synthesizing the αGal epitope on cellular, as well as on viral carbohydrate chains (GEYER et al., 1984; REPIK et al., 1994). Consequently, these viruses, which infected cells containing active *GGTA1* had multiple αGal epitopes on their envelope gylcoproteins (GALILI et al., 1996; PATIENCE et al., 1997; DURRBACH et al., 2007). Thus, the virulent enveloped viruses, provided with αGal epitopes by the ancestral parental primate population were then neutralized and destroyed by the offspring that lost this epitope and produced anti-Gal antibodies, in contrast to their parental generation, which were susceptible to the viruses and underwent extinction.

Evidently, the possibility of genetically modifying genomes facilitates the recapitulation of an evolutionary process. The tailoring of donor pigs for xenotransplantation by disruption of genes involved in the glycosylation process can be seen as the bringing together of genetic

constellations that drifted apart by natural selection. It is of note, however, that evolutionary fundamental events such as the deletion of the *GGTA1* gene in old-world primates are rare and manifest within generations. In much shorter terms, evolution is quite stable. This is illustrated by the stable inheritance of the hCD46 and the hTBM transgenes in our breeding herd for meanwhile 8 generations. Moreover, the expression profile of the transgenes, and thus, the quality of the donor pigs remains stable through many generations of breeding (LANGIN et al., 2018). Therefore, it is not necessary to produce xenotransplantation donor animals by SCNT, but to provide them in a rather easy way, by breeding.

6 Summary

With the growing demand for deceased organ donors and the steady increase of an aging population, a solution to the organ shortage has to be found. Xenotransplantation can offer a feasible solution within the near future, based on the potential, documented in recent preclinical trials. To further promote these perspectives, the aim of this doctoral thesis was to evaluate and improve the hygiene status of donor pigs for preclinical trials of collaboration projects embedded in the CRC TRR 127.

Major parts of my work on hygiene sanitation have been done during the population of a new facility, the Center of innovative Medical Models (CiMM), at the Chair of Molecular Animals Breeding and Biotechnology (MABB) (EGERER et al., 2018). For this, pregnant DPF sows from a barrier facility were introduced to the new facility, CiMM, and quickly removed after farrowing, with the piglets only being able to suckle colostrum every 2 hours for 24 hours under supervision, to avoid any further contact with the mother sows. The motherless reared female piglets became the foster mothers of SCNT and IVF derived genetically-multimodified pig lines, which were introduced solely by embryo transfer (ET). With this approach we were able to establish a PCMV-free pig facility. But not only screening for PCMV was done, in the progress of the herd sanitation we screened for other exogenous pathogens, as listed in **Table 12** .

Another step for the provision of safe donor pigs is the continuing assessment of potential new threats for xenograft recipients, as it was exemplified by the detection of PCV3 in our herd and in four baboons, after being transplanted with infected porcine hearts (KRUGER et al., 2019). Permanent maintenance of a breeding herd for xenotransplantation donors is, however, necessary for the constant supply of safe donor pigs, which is one of the essential prerequisites for robust pre-clinical studies, as it has been recently documented in a groundbreaking pig-to-baboon heart transplantation study (LANGIN et al., 2018). The continuous production of pigs by breeding was also a good opportunity to address another safety aspect in xenotransplantation, the abundance of PERVs, specifically of the subfamily C. I was clearly able to document that none of the PERV-C proviruses was abundant in all animals and that the proviruses are inherited in a Mendelian manner. In some of the animals this resulted in a PERV-C free status, suggesting that breeding-out of PERV-C is possible, albeit this will take some time, due to the low number of animals being produced and the long

generation time of pigs. Another remarkable finding was, that there are truncated PERV-C proviruses, which have very likely accumulated in the pig genome by retroviral hitchhiking, as the respective proviruses were flanked by 4 bp duplications, a very characteristic feature of retroviral infection. Finally, the presence of breeding herds for xenotransplantation facilitated also the ability to gain materials for other projects. A good example for that, was a study on immunosurgery of blastocysts, which is profoundly promoted by serum the from GTKO pigs, which contains high levels of anti-Gal-antibodies to induce complement-mediated lysis of blastocysts, expressing the αGal epitope (KUROME et al., 2019).

Thus, my doctoral thesis illustrates the high relevance of continuous production of genetically modified pigs for xenotransplantation by breeding and documents the ability to remove exogenous as well endogenous pathogens from an existing herd. These findings will be essential aspects for paving the way towards eventually supplying donor pigs also for clinical research.

7 Zusammenfassung

Bereitstellung sicherer Spenderschweine für die Xenotransplantation

Aufgrund des steigenden Bedarfs an Organen verstorbenen Organspendern und einer stetig wachsenden und alternden Bevölkerung, muss eine Lösung für diese Spenderorganknappheit gefunden werden. Die Xenotransplantation könnte hierfür eine machbare Lösung in naher Zukunft sein, was sich auf den Leistungen begründet, die in der aktuellen vorklinischen Forschung dokumentiert wurden. Um diese Aussichten weiter voranzutreiben, war das Ziel dieser Doktorarbeit, den Hygienestatuts von Spenderschweinen für präklinische Forschungsprojekte innerhalb des Sonderforschungsbereich 127 zu evaluieren und zu verbessern.

Große Teile meiner Arbeit an der Hygienesanierung, fanden während der Neubesiedlung unserer neuen Stallanlage, dem „Center of innovative Medical Models (CiMM) am Lehrstuhl für Molekulare Tierzucht und Biotechnologie (MABB), statt (EGERER et al., 2018). Dafür wurden trächtige DPF Sauen von einer Stallanlage mit strikt geregeltem und eingeschränktem Zugang, in unsere neue Anlage, CiMM, gebracht und sofort nach dem Abferkeln wieder entfernt, wobei die Ferkel für 24 Stunden alle 2 Stunden Kolostrum unter Aufsicht trinken konnten, um jeglichen weiteren Kontakt mit den Muttersauen zu vermeiden. Die mutterlos-aufgezogenen weiblichen Ferkel wurden Ziehmütter von SCNT und IVF generierten, genetisch-multimodifizierten Schweinelinien, die ausschließlich durch Embryotransfer (ET) eingebracht wurden. Durch diese Herangehensweise konnten wir einen PCMV-freien Schweinestall aufbauen. Außerdem wurde nicht nur auf PCMV untersucht, sondern während der Herdenerneuerung und -bereinigung, wurde auch auf andere exogene Pathogene untersucht, die in **Table 12** aufgeführt sind.

Der nächste Schritt, bei der Bereitstellung von sicheren Spenderschweinen, ist die fortlaufende Einschätzung potenzieller neuer Gefahren für Xenotransplantatempfänger, wie es der Nachweis von PCV3 in unserer Herde und in vier Pavianen, nach der Transplantation mit infizierten Schweineherzen, veranschaulicht hat (KRUGER et al., 2019). Die andauernde und fortlaufende Instandhaltung einer Zuchtherde von Spenderschweinen für die Xenotransplantation ist, trotz allem und gerade für die konstante Versorgung mit sicheren Spenderschweinen, eine notwendige, essentielle und unabdingbare Grundvoraussetzung für

robuste präklinische Studien, wie es kürzlich erst gezeigt wurde, in einer wegweisenden Schwein-zu-Pavian Herztransplantationsstudie (LANGIN et al., 2018). Die ständige Nachzucht von Schweinen, durch Unterhaltung einer Zuchtherde, war auch eine gute Möglichkeit, um einen anderen Sicherheitsaspekt der Xenotransplantation, die Anzahl von PERVs, im Speziellen von der Unterfamilie C, zu behandeln. Es war mir möglich klar zu dokumentieren, dass keiner der PERV-C Proviren in allen Tieren vorkam und dass die Proviren nach den Mendelschen Regeln vererbt werden. Bei manchen Tieren führte das zu einem PERV-C freien Status, was die Schlussfolgerung zulässt, dass man PERV-C durch Zucht elimieren kann, wenngleich das einige Zeit dauern wird, wegen der geringen Tierzahlen die produziert werden und der langen Generationsdauer von Schweinen. Eine weitere hervorzuhebende Erkenntnis war, dass es „kaputte“ PERV-C Proviren gibt, die sich höchstwahrscheinlich als retroviraler Anhalter/Mitfahrer im Schweinegenom angereichert haben, da die betreffenden Proviren von 4bp Verdoppelungen flankiert waren, was ein charakteristisches Zeichen für eine retrovirale Infektion ist. Zuletzt hat die Existenz von Zuchtherden für die Xenotransplantation die Möglichkeit gegeben, Material für andere Projekte zu sammeln. Ein gutes Beispiel ist die Studie über „Immunchirurgie“ an Blastozysten, die ungemein vom Serum der GTKO Schweinen profitiert hat, da das Serum hohe Level an anti-Gal-Antikörpern enthält, welche die Komplement-mediierte Lyse von Blastozysten, die αGal-Oberflächenepitope exprimieren, induzieren (KUROME et al., 2019).

Folglich veranschaulicht meine Doktorarbeit die hohe Relevanz der kontinuierlichen Produktion genetisch-modifizierter Schweine für die Xenotransplantation durch Zucht und belegt, dass man sowohl exogene, als endogene Pathogene von einer bereits existierenden Herde beseitigen kann. Um den Weg für eine mögliche Bereitstellung von Spenderschweinen auch für die klinische Forschung, zu ebnen, werden diese Erkenntnisse essentielle Gesichtspunkte sein.

8 Literature

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9 Figures

10 Tables

11 Appendix

Pig	Day 1	Day 6	Day 12	Day 26	Day 50	Day 73	Day 135	Day 230	> 1 Year †
#1	–§	–	–	–	–	dead			
#2	–	–	–	–	–	–	–	dead	
#3	–	dead							
#4	–	–	–	–	–	–	–	–	dead
#5	–	dead							
#6	–	–	–	–	–	–	–	–	–
#7	–	–	–	–	–	–	–	–	–
#8	–	–	–	–	–	–	–	–	–
#9	–	–	–	–	–	–	–	–	dead
#10	–	–	–	–	–	–	–	–	n.d. ‡
#11	–	dead							
#12	dead								
#13	–	–	–	–	–	–	–	dead	
#14	–	dead							
#15	–	–	–	–	–	–	–	–	–
#16	–	dead							
#17	–	–	–	–	–	–	–	–	dead
#18	–	–	–	–	–	–	–	–	dead
#19	–	–	–	–	–	dead			
#20	–	dead							
#21	–	–	–	–	–	–	–	–	–
#22	–	–	–	–	–	–	–	–	–
#23	–	–	–	–	–	–	–	dead	
#24	–	–	–	–	–	–	–	–	n.d.
#25	–	–	–	–	–	–	–	dead	
#26	–	–	–	–	–	–	–	–	dead
#27	–	–	–	–	–	–	–	dead	
#28	–	–	–	–	–	dead			
#29	–	–	–	–	–	–	–	–	–
#30	–	–	–	–	–	–	–	–	n.d.
#31	–	–	–	–	–	–	–	–	–
#32	–	–	–	–	–	dead			
#33	–	–	–	–	–	–	–	dead	
#34	–	–	–	–	–	–	–	dead	
#35	–	–	–	–	–	dead			
#36	–	–	–	–	–	–	–	dead	
#37	–	–	–	–	–	–	–	–	–
#38	–	–	–	–	–	–	–	–	n.d.
#39	–	–	–	–	–	–	–	–	n.d.
#40	–	–	–	–	–	–	dead		
#41	–	–	–	–	–	–	–	–	n.d.
#42	–	–	–	–	–	–	–	–	–

#43	–	–	–	–	–	–	–	–	–
#44	–	–	–	–	–	–	–	–	–
#45	–	–	–	–	–	–	–	–	–
#46	–	–	–	–	–	–	–	–	–
#47	–	dead							
#48	–	–	–	–	–	–	–	–	–
#49	–	–	–	–	–	–	–	–	n.d.
#50	–	–	–	–	–	–	–	–	–
#51	–	dead							
#52	–	–	–	–	–	–	–	–	–
#53	–	–	–	–	–	–	–	–	–
#54	–	–	–	–	–	–	–	–	–
#55	–	–	–	–	–	–	–	–	dead
#56	–	–	–	–	–	–	–	–	–
#57	–	dead							
#58	–	–	–	–	–	–	–	–	–
#59	–	–	–	–	–	dead			
#60	dead								
#61	–	–	–	–	–	–	dead		
#62	–	–	–	–	–	–	–	–	n.d.
#63	–	–	–	–	–	dead			
#64	–	–	–	–	–	–	–	–	–
#65	–	–	–	–	–	–	–	–	–
#66	–	–	–	–	–	–	–	–	–
#67	–	–	–	–	–	–	–	–	–
#68	–	–	–	–	–	–	–	–	–
#69	–	–	–	–	–	–	–	–	–
#70	–	–	–	–	–	–	–	–	–
#71	–	–	–	–	–	dead			
#72	–	–	–	–	–	–	–	–	–
#73	–	–	–	–	–	–	–	–	dead
#74	–	–	–	–	–	–	–	–	n.d.
#75	–	–	–	–	–	–	dead		
#76	–	–	–	–	–	–	–	–	dead
#77	–	–	–	–	–	–	–	–	n.d.
#78	–	dead							
#79	–	–	–	–	–	–	–	–	–
#80	–	dead							
#81	–	dead							
#82	–	dead							
#83	–	dead							
#84	–	–	–	–	–	–	dead		
#85	–	–	–	–	–	–	dead		
#86	–	dead							
#87	–	–	–	–	–	–	–	–	–
#88	dead								
#89	dead								

#90	–	dead						
#91	–	–	–	–	–	–	–	n.d.

† tested on DNA extracted directly from tissue, blood or serum without PBMC cultivation

‡not determined

§negative result

SUPPLEMENTARY TABLE 1 PCMV screen of all F1 generation offspring.

F1 generation offspring was screened at regular intervals over the whole observation period of 14 months. All F1 animals are negative for PCMV at all tested time points. (Taken from EGERER et al., 2018)

number	chr1:20		chr1:41		chr1:3		chr2:10		chr4:135		chr5:23		chr6:41		chr7:18		chr11:29		chr13:104		chr14:62		Chr16:79		chrX:14		scf200	
	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV
1410	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
1481	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
1511	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
1621	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
1622	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
1761	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
1929	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
1938	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
1974	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
2017	pos	pos	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	pos
2041	neg	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
2074	pos	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
2909	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
3360	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos
3603	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos
4667	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4679	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4682	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4686	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4688	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4689	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
4775	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg
4791	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
5004	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
5006	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
5411	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
5426	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
6182	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg

6183	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
6184	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
6185	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
6186	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
9781	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
9864	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
X101	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
X102	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
X126	pos	pos	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos
X127	pos	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
X129	pos	pos	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos
X149	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos
X155	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos
Y108	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos
1476	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos
4504	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg
4505	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg
1044	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	n.d.	neg	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	neg
3043	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	n.d.	pos	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	pos
3047	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	n.d.	pos	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	pos
9753	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	n.d.	neg	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	pos
9781	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	n.d.	pos	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	neg
9874	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	n.d.	neg	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	neg
9875	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	neg	neg	pos	neg	n.d.	neg	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	pos
9943	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	n.d.	pos	pos	neg	n.d.	n.d.	pos	neg	pos	neg	pos	neg
1476-Z	pos	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	n.d.	neg	pos	pos	n.d.	n.d.	pos	pos	pos	neg	pos	pos
4504-Z	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg	pos	neg	neg	pos	pos	pos	n.d.	neg	pos	neg	n.d.	n.d.	pos	pos	pos	pos	pos	neg
9869	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	n.d.	n.d.	pos	neg	n.d.	n.d.	neg	neg	pos	neg	pos	neg

Table 13 All important animals from the pedigree shown in **Figure 7** were examined for the presence of the 11 PERV-C and the 3 PERV-C-like proviral remnants.

number	chr1:20		chr1:41		chr1:3		chr2:10		chr4:135		chr5:23		chr6:41		chr7:18		chr11:29		chr13:104		chr14:62		Chr16:79		chrX:14		scf200	
	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV	LTR	PV
6202	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	WT	pos
6203	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	neg	pos	WT	pos
6204	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6205	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	pos	pos	neg	wt/lt	neg
6206	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6207	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	neg	pos	wt/lt	neg
6208	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	pos	WT	pos
6209	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	pos	WT	pos
6210	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	WT	pos
6211	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	WT	pos
6246	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6247	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6248	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	neg	pos	neg	pos	pos	neg	pos	neg	neg	neg	pos	neg	wt/lt	neg
6249	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6250	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6251	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	neg	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6252	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	pos	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6253	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6254	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6255	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	WT	neg
6256	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6257	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6258	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	WT	neg
6259	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6260	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6261	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	neg	neg	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	WT	neg
6262	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos

6263	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6327	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6328	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6329	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6330	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6331	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6332	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6333	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6334	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6335	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	neg	WT	pos
6336	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	neg	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6354	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	neg	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6355	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	neg	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6356	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6357	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6358	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6359	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6360	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6361	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6362	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6391	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6392	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6393	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6394	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6395	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6396	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6467	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6468	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6469	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6470	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg

6471	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6472	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6473	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6474	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6475	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6492	pos	neg	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	neg	neg	pos	neg	pos	neg	WT	pos
6493	pos	pos	pos	neg	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6494	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6510	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6511	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6512	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6513	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6514	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6515	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6516	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6517	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	pos
6518	pos	neg	neg	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6519	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	neg
6520	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	wt/lt	pos
6521	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6522	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6542	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6543	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6544	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6545	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6546	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6547	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6548	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6549	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6550	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos

6551	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6552	pos	pos	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	WT	pos
6553	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6554	neg	neg	pos	neg	pos	pos	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg
6555	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	WT	neg

Table 14 Additionally to the important animals from the pedigree of **Figure 7**, all offspring was examined for the presence of the 11 PERV-C and the 3 PERV-C-like proviral remnants, too.

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