Genetic diversity, growth and heart function of Auckland Island pigs, a potential source for organ xenotransplantation

Von Andreas Lange

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Von Andreas Lange

aus Herford

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Veterinärwissenschaftlichen Department

der Ludwig-Maximilians-Universität München

Lehrstuhl für Molekulare Tierzucht und Biotechnologie

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
B4GALNT2/ *B4GALNT2L*	β-1,4-N-acetyl-galactosaminyltransferase 2
BiVAD	Bi-ventricular assist device
BMI	Body mass index
BSA	Body surface area
CD39	Ectonucleoside triphosphate diphosphohydrolase-1
CD46	Membrane cofactor protein
CD47	Leukocyte surface antigen CD47
CD55	Complement decay accelerating factor
CiMM	Centre for Innovative Medical Models
CMAH	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats – CRISPR associated protein 9
DNA	Deoxyribonucleic acid
DPF	Designated pathogen-free
EAA	Erythrocyte antigen A
EBV	Epstein-Barr virus
EF	Ejection fraction
ELISA	Enzyme linked immunosorbent assay
EPCR	Endpoint PCR
FDA	Food and Drug Administration
FS	Fractional shortening
GHR	Growth hormone receptor
GM	Genetically multi-modified
HEV-3	Hepatitis E virus 3
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMOX1	Heme oxygenase 1
HTK	Histidine, tryptophane, ketoglutarate solution
IL-6	Interleukin 6
IPD	Immuno-Polymorphism Database
LA	Left atrium
LAX	Long axis
LMU	Ludwig-Maximilians-Universität München
LV	Left ventricle

LVAD	Left ventricular assist device
LVEF	Left ventricular ejection fraction
MCS	Mechanical circulatory support
MHC	Major histocompatibility complex
Neu5G	N-glycolylneuraminic acid
NHP	Nonhuman primate
PBMC	Peripheral blood mononuclear cells
PCMV	Porcine cytomegalovirus
PCR	Polymerase chain reaction
PERV	Porcine endogenous retrovirus
PHLV-1	Porcine Lymphotropic Herpesvirus-1
PHM	Predicted heart mass
PRV	Porcine roseolovirus
PTLD	Post transplantation lymphoproliferative disorder
RA	Right atrium
Rh	Rhesus factor
RNA	Ribonucleic acid
ROH	Runs of homozygosity
RT-PCR	Real time PCR
RV	Right ventricle
RVAD	Right ventricular assist device
SCNT	Somatic cell nuclear transfer
SLA	Swine leukocyte antigens
SPF	Specified pathogen-free
TBM	Thrombomodulin
TNFα	Tumor necrosis factor alpha
tPA-PAI-1	Tissue plasminogen activator and plasminogen activator inhibitor 1 complexes
TTSuV	Torque teno sus virus
UNOS	United Network for organ sharing
US	United States of America
αGal	Galactose-α(1,3)-galactose

I. INTRODUCTION

Heart failure is an ever growing epidemic, affecting roughly 1-5% of the population (AUTHORS/TASK FORCE et al., 2022). Though new treatment options have emerged, the gold standard for advanced heart failure remains heart transplantation (AUTHORS/TASK FORCE et al., 2022). The outcome of a heart transplantation has also improved over the years and the 5-year survival is at 92,1% in some conditions (HSICH et al., 2022), but only 61,2% of waitlist patients get transplanted and the average time on the waiting list is 196 days (BAKHTIYAR et al., 2020). Many attempts have been made to alleviate the situation, but the problem of a severe shortage in donor organs remains to this day (AWAD et al., 2022).

Xenotransplantation is the transplantation of cells, tissue or organs from one species to another (DENNER, 2020b). So far there have been two compassionate pig to human heart transplantations with promising results (GRIFFITH et al., 2022; UNIVERSITY OF MARYLAND, 2023). For this achievement a great deal of scientific research was necessary. First attempts using nonhuman primates as organ donors failed. Due to infectious risks and ethical concerns a different species was needed (ALLAN, 1998). Pigs presented as a good choice, due to similarities in anatomy and size and their high reproductive capacity in comparison to nonhuman primates (NHPs) (REICHART et al., 2021).

The generation of genetically multi-modified pigs was an important step to overcome immune barriers and prevent natural rejection mechanisms of humans towards porcine tissue. The elimination of three carbohydrate antigens αGal, Neu5G and β4Gal prevented hyperacute rejection of the xenograft. Introducing human complement regulatory proteins, like CD46 and CD55, to prevent complement activation, and human thrombomodulin to prevent coagulopathy were other important steps. Graft overgrowth was either prevented through medical treatment or by a knockout of the growth hormone receptor (*GHR*) gene, reducing the pigs and their organs in size (REICHART et al., 2023).

Another issue is microbiological safety, with screening and eradication methods having been postulated for most viruses. However, porcine endogenous viruses (PERV) are still a concern, as they are integrated in the genome of almost any pig line (DENNER, 2020a).

The aim of this thesis is to explore Auckland Island pigs as a potential genetic background for xenotransplantation. Through their natural traits, such as similar size to humans and being naturally free of PERV-C, they could prove to be superior to conventional pig breeds and other minipig lines.

II. REVIEW OF THE LITERATURE

1. Heart Failure

Heart failure was declared an epidemic throughout the entire world in 1997 (BRAUNWALD, 1997) and is currently affecting 63 million people worldwide (GROENEWEGEN et al., 2020). It is associated with a shortened lifespan, poor quality of life and a substantial burden to the healthcare system (SHAHIM et al., 2023). Although treatment has improved in the last years, the number of cases is continuously growing. Causes are an aging population, better treatment of cardiovascular disease and a changing lifestyle in the developing countries (HEIDENREICH et al., 2013; GROENEWEGEN et al., 2020; EMMONS-BELL et al., 2022). Data on prevalence and incidence varies, since different studies use a variety of parameters to measure heart failure. The incidence in Europe is 5/1000 in adults and the prevalence is between 1-2% in adults (AUTHORS/TASK FORCE et al., 2022). One-year case fatality rages from 4% to 45% with an average at 33% (EMMONS-BELL et al., 2022). Patients with heart failure are hospitalized once a year on average (GERBER et al., 2015).

Heart failure is a complex of pathologies that arise from structural or functional abnormalities of the heart. This results in elevated intracardial pressure or reduced cardiac output (AUTHORS/TASK FORCE et al., 2022). Often heart failure is characterized by left ventricular ejection fraction and classified in heart failure with reduced ejection fraction (LVEF $\leq$ 40%), heart failure with mildly reduced ejection fraction (LVEF 41-49%), and heart failure with preserved ejection fraction (LVEF $\geq$ 50%) (AUTHORS/TASK FORCE et al., 2022). A variety of diagnostic tools has been proposed to predict heart failure, but standards vary between studies (GROENEWEGEN et al., 2020; AUTHORS/TASK FORCE et al., 2022). Comorbidities like hypertension, diabetes mellitus or kidney disease make the occurrence of heart failure more likely. Often symptoms are breathlessness, fatigue and ankle swelling, which are not specific enough on their own to make the diagnosis. According to the European Society of Cardiology, electrocardiogram, natriuretic peptide levels, blood chemistry, echocardiography, and chest X-ray are recommended for the diagnosis (AUTHORS/TASK FORCE et al., 2022). Echocardiography is a key diagnostic tool, which can determine ejection fraction but also cardiac dimensions and pathologies (LANG et al., 2015).

Often heart failure patients come into a condition of advanced heart failure, despite guideline directed medical therapy (CRESPO-LEIRO et al., 2018; TRUBY & ROGERS, 2020). The criteria defining advanced heart failure are severe persistent symptoms, severe cardiac dysfunction, episodes of pulmonary or systemic congestion, and severe impairment of exercise capacity. Severe cardiac dysfunction comprises reduced LVEF (<30%), isolated RV failure, non-operable severe valve abnormalities, congenital abnormalities, persistently high natriuretic peptides, severe diastolic dysfunction, or severe LV structural abnormalities (CRESPO-LEIRO et al., 2018). Advanced heart failure can be treated by long-term mechanical circulatory support (MCS) or heart transplantation. Short-term drug therapies such as inotropes can be considered, although there might be contraindications (VISHRAM-NIELSEN et al., 2022). Also, temporary MCS devices can bridge the time until heart transplantation or a long-term MCS can be implanted (KURIHARA et al., 2018). They are relatively simple to install and their technology has evolved rapidly over the past years. From being exclusive as a rescue strategy, there are more indications for temporary MCS, such as catheterization laboratory, electrophysiology suite, operating room and intensive care unit. Therefore, they need to be selected for the type of support and time needed. The use of temporary MCS can be considered in patients with high surgical risk to reach clinical stability. It is also used in primary allograft dysfunction, to allow myocardial recovery and adequate end organ perfusion (SALTER et al., 2023).

2. Long-term mechanical circulatory support (MCS)

In advanced heart failure, long-term mechanical circulatory support (MCS) devices gain in relevance as the number of patients is increasing but the number of transplant organs remains stable on a low level. MCS devices can either be used as bridging until a heart is available for transplantation or as a destination therapy, for the rest of the patient's life (POTAPOV et al., 2019; FELIX et al., 2020). As most patients with long-term MCS never get transplanted, the bridging technology often leads to destination therapy. In destination therapy the patient can be managed in an out of hospital setting.

Currently most long-term devices are intracorporeal and left ventricular assist devices (LVAD) as seen in Figure 1. The pumps developed from pulsatile flow

devices, to continuous flow axial devices with contact bearings and seals. As bearings increase the occurrence of thrombosis the third generation of pumps are centrifugal continuous flow pumps without contact bearings or seals (KIM et al., 2018). In these pumps the flow is generated by a centrifugal rotor, that is levitated in an electromagnetic field with no contact to the housing. This leads to minimal shear stress compared to an axial pump (ATTI et al., 2022). The percentage of these pumps with full magnetic levitation has increased in the last years and the majority of implants utilized this technology in 2019 (DE BY et al., 2022).

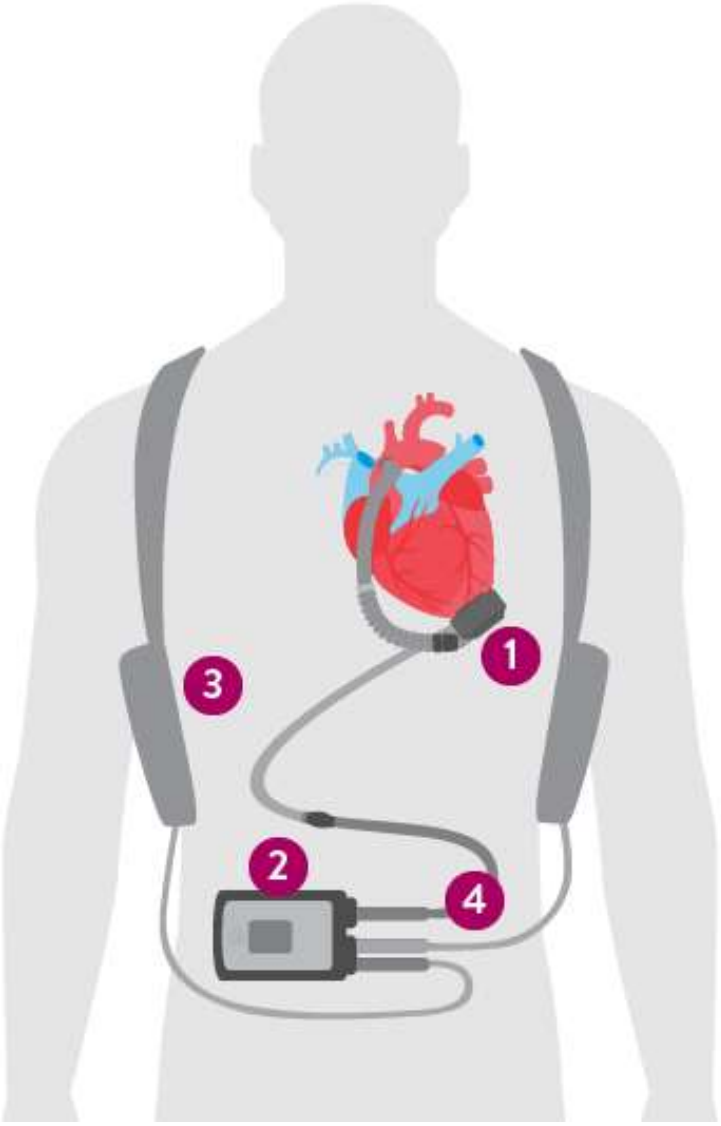


Figure 1: 1. LVAD Heart Mate 3, magnetically levitated continuous flow pump; 2. Controller including emergency battery; 3. Batteries; 4. Modular driveline (GMBH, 2024a)

Right heart failure occurs in 10-30% of cases and can either be accommodated by a biventricular assist device, or a total artificial heart (FARAG et al., 2021). A biventricular assist device (BiVAD) bypasses the heart in both directions. Also, continuous flow pumps are used, which improved the outcome for the patient, with a 12-month survival of 58.5% (FARAG et al., 2021). The total artificial heart completely replaces the heart by a pneumatically powered device. This option is only available in patients with a large chest cavity (>10 cm between the 10^{th} thoracic vertebra and sternum) (COOK et al., 2015). The 12-month survival after implantation is at 53% (ARABIA et al., 2018).

According to the European Registry for Patients with Mechanically Circulatory Support (EUROMACS) there is an overall 5-year mortality of 55%, 25% of patients get transplanted and 2% get weaned of support (DE BY et al., 2022). Common complications during treatment are bleeding, infections, neurological events, pump thrombosis or unexpected readmissions. Contraindications for MCS are irreversible neurological or neuromuscular disorders, kidney failure, liver failure, mental illness or instability, medication non-adherence or severe RV dysfunction with no option of RVAD support (ATTI et al., 2022).

3. Heart transplantation

Currently heart transplantation remains the gold standard in the treatment of advanced heart failure (MEHRA et al., 2016; CRESPO-LEIRO et al., 2018; TRUBY & ROGERS, 2020; AUTHORS/TASK FORCE et al., 2022). Since the first heart transplantation in 1967 by Barnard, techniques have improved tremendously and the 5-year survival time ranges between 92,1% and 74,3% depending on the pathology causing heart failure (HSICH et al., 2022). In the past years, the number of heart transplantations has stabilized at 8,000 per year worldwide (TRANSPLANTATION, 2023).

One major improvement was the development of cyclosporin, which made a well-tolerated immunosuppressive therapy possible (CALNE et al., 1979). Also, MCS devices and better treatment of advanced heart failure patients have led to an increase in 1-year survival on the transplant waiting list from 34.1% (1987-1990) to 67.8% (2011-2017) (BAKHTIYAR et al., 2020). In 2018, a new allocation

system was devised by the United Network for Organ Sharing (UNOS) (MEYER et al., 2015). It was meant to prioritize patients on temporary MCS given their immediacy of treatment. This led to a decrease in days on the waiting list, although the total number of heart transplantations didn't change significantly (GANAPATHI et al., 2023).

Risk factors in the recipients are obesity, pulmonary arterial hypertension, renal dysfunction, prior sternotomy, prolonged ventilator support or intensive care unit stay and MCS use. Gender mismatch and increased donor age also worsen the outcome of heart transplantation (AWAD et al., 2022). To make more donor organs available, hearts of patients after circulatory death were also utilized in the United States (QUADER et al., 2013). Also, an improved treatment of hepatitis C with direct acting antivirals has made the use hearts from infected patients feasible (HUCKABY et al., 2021).

Possible complications of heart transplantation are primary graft dysfunction, which can lead to early graft failure. This is defined as graft failure within in the first 30 days. This is the most feared complication with an incidence of 3.8% from 2005-2013 with a 96.3% mortality rate (LUND et al., 2015; MCCARTNEY et al., 2017; AWAD et al., 2022). Another complication is cardiac allograft vasculopathy, which is a progressive vascular occlusive disease affecting the coronary vessels. With an incidence of 7.8% at 1 year its relevance increases to 50% at 10 years (LUND et al., 2015). Chronic kidney disease, neurological events and changes in pulmonary function are also common side effects of heart transplantation (MCCARTNEY et al., 2017).

3.1. Criteria for organ donation

The criteria for organ donation have last been updated in 2016. Patients should be considered for transplantation if cardiopulmonary stress testing shows an oxygen consumption of <14 ml/kg/min in the presence of a cardiac synchronization therapy device. This should be combined with a heart failure survival prognosis score to determine the prognosis of the patient. The 1-year survival should be above 80%. Diagnostic right heart catheterization should be performed to assess pulmonary hypertension and progression of the heart failure (MEHRA et al., 2016). Irreversible pulmonary hypertension is a contraindication for heart transplantation.

Comorbidities, such as age over 70 years, obesity (BMI >35 kg/m^2), diabetes mellitus with end organ damage, irreversible renal disease (eGFR <30 ml/min/1.73 m^2), clinical severe cerebrovascular disease or mental illness pose as relative contraindications (MEHRA et al., 2016; AWAD et al., 2022).

3.2. Size matching

Matching organ size between donor and recipient is crucial as mismatched organs are correlated to a significantly higher chance of graft failure. Several parameters have been used like weight, height, BMI or BSA, none of which could give an accurate prediction of organ size on their own (KHUSH et al., 2019). Therefore, the International Society for Heart and Lung Transplantation recommends using the predicted heart mass (PHM) as more accurate parameter for size matching, incorporating height, weight, age and sex altogether (KHUSH et al., 2019). For a good size match the PHM should not vary more than 20% between donor and recipient.

3.3. Organ shortage

The crucial issue in orthotropic heart transplantation today is the shortage of donor organs. There are far more patients on the waiting list than organs available. In March 2024 there were 3,436 people on the waiting list in the US, while there were only 812 heart transplants performed (ADMINISTRATION, 2024). Between 2011 and 2017, 7% of patients died on the waiting list, while only 61.2% got transplanted. The mean waiting time for a transplant was 196 days (BAKHTIYAR et al., 2020). In the US, there have been some approaches to alleviate the situation, such as the use of organs from hepatitis C infected patients and donations after circulatory death (AWAD et al., 2022). Other possibilities are in educating health care professionals and in breaking down bureaucratic barriers in the process (LEWIS et al., 2021). Further it is discussed to limit the pool of recipients by increasing their standards to generate a better outcome (LEWIS et al., 2021). The opt-out solution for organ donors does not seem to increase donor numbers on a great scale (ETHEREDGE, 2021).

4. Xenotransplantation

By definition, xenotransplantation is any procedure that involves the transplantation, implantation, or infusion into a human recipient of live cells, tissues, or organs from an animal source. It also includes human body fluids, cells, tissues, or organs that have had *ex vivo* contact with live animal cells, tissues, or organs (DENNER, 2020b). First attempts to implant a chimpanzee heart into man were made in 1964 by Hardy (HARDY et al., 1964). Unfortunately, the heart didn't support circulation in the recipient. Further attempts have been made with different species using either orthotropic or heterotopic heart transplantation with limited success (TANIGUCHI & COOPER, 1997). Preclinical studies of transplanting pig hearts to baboons in Munich led to great advances in the survival time of the recipients (LANGIN et al., 2018; REICHART et al., 2020) and were a prerequisite of the first successful pig to human xenotransplantation. The procedure took place on 57-year-old David Bennett in Maryland on January 7th 2022 (GRIFFITH et al., 2022; HAWTHORNE, 2022; REARDON, 2022; MOHIUDDIN et al., 2023) with a survival time of 60 days after transplantation. The second attempt was also made at the University of Maryland on 58-year-old Laurence Faucett on September 20th 2023 (UNIVERSITY OF MARYLAND, 2023) who survived 6 weeks after the surgery.

5. Species for xenotransplantation

5.1. Nonhuman primates

Nonhuman primates (NHPs) such as chimpanzee or baboon were already used as organ donors for xenotransplantation. Apart from a young girl, that survived for 20 days (BAILEY et al., 1985) these attempts were not successful. Ethical concerns and poor acceptance for these attempts in the general population have impeded the use of these species (PRESCOTT, 2010; CENGIZ & WAREHAM, 2020). The risk of infection through the xenograft is also high, since NHPs are immunologically close to humans and share a variety of viruses (ALLAN, 1998).

5.2. Pigs

5.2.1. Anatomy and physiology

As an alternative to NHPs, pigs are now the favored source of xenotransplantation hearts. Their heart size and function are similar to humans. There are differences in the heart shape due to the quadruped stands of pigs. The cranial and caudal caval veins open at right angles into the atrium while in man to posterior and inferior caval veins are in line. A very prominent azygos vein drains directly in the coronary sinus. In humans this is not present. The right atrium is only drained by 2 pulmonary veins. In man there are usually 4 pulmonary veins respectively. Aortic-mitral fibrous continuity was reduced in the outlet component of the porcine left ventricle, with the majority of the aortic valve being supported by the muscular portion of the left ventricle. Another prominent feature is the septomarginal trabecula in the right ventricle, which is much broader in pigs than in humans and carries Purkinje fibers from the septum to the free wall (CRICK et al., 1998; LELOVAS et al., 2014).

Physiologically pigs vary in hemodynamics according to their race, age, and weight. As farm pigs show pressures resembling human standard values, minipigs are at a systolic pressure of around 60 mmHg. Electrophysiologically there are some differences like a faster sinus rhythm, shorter PR-intervals and simultaneous activations of the endocardium and epicardium (LELOVAS et al., 2014).

Due to the approximation of hemodynamic values, the similarity of their coronary circulation, similar valve anatomy and their size, pigs do generate viable hearts for xenotransplantation. Their anatomical differences need to be considered during the transplantation procedure (CRICK et al., 1998; LELOVAS et al., 2014).

Growing domestic pigs do have the same ratio of heart weight to body weight (5g/kg) until they reach about 150 kg. Humans tend to have an average heart weight from 156 to 422 g in women (MOLINA & DIMAIO, 2015) and from 188 to 575 g in men (MOLINA & DIMAIO, 2012). In comparison modern farm pigs have an adult weight over 200 kg and have heart weights of over 600 g on average (VAN ESSEN et al., 2018).

5.2.2. Availability of organs

Swine have a high reproductive capacity, with 12.65 live born piglets per litter and 2.39 litters per year on average in modern farm breeds (PIEROZAN et al., 2020). Also, pigs have a short generation time of approximately one year. Therefore, a large number of organs can be made available in a short amount of time. On the other hand, they have a life expectancy of 15-20 years depending on the breed. This suggests a possible longevity of the pig organs in clinical use (REICHART et al., 2021).

6. Genetic modification of source pigs

6.1. Elimination of carbohydrate antigens

A major obstacle in using porcine tissues for xenotransplantation is the hyperacute immune reaction of the recipient. This reaction is mainly triggered by antibodies against galactose-α(1,3)-galactose (αGal), N-glycolylneuraminic acid (Neu5Gc) and a glycan corresponding to the human Sd(a) blood group antigen (REICHART et al., 2021). Humans do not have these antigens, but they develop antibodies against them during infancy in reaction to colonization of the bowel with certain stems of bacteria (COOPER et al., 1993). NHPs have Neu5Gc and develop antibodies only against αGal and Sd(a). After xenotransplantation of wild-type pig organs to humans or NHPs, graft failure occurs within minutes to hours, due to venous thrombosis, loss of vascular integrity, hemorrhage, edema and innate immune cell infiltration (REICHART et al., 2023). To overcome the hyperacute rejection, pigs with a knockout of the α-1,3-galactosyltransferase gene (*GGTA1*) were generated (PHELPS et al., 2003). The xenotransplantation of *GGTA1*-knockout pig hearts led to increased graft survival times of 2–5 months in baboons (COOPER et al., 2007). Subsequently, pigs with inactivated cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*) and β-1,4-N-acetyl-galactosaminyltransferase 2 (*B4GALNT2*/*B4GALNT2L*) genes were generated to eliminate Neu5Gc and Sd(a) (ALI et al., 2024).

6.2. Complement regulation

The next step was suppressing other immunologic factors like the humoral reaction through the complement system. Regulatory proteins moderate the complement cascade and prevent damage to the host tissue. As porcine complement regulatory proteins have no effect on the human complement system and suppression of the

complement system in the recipient is not preferable, a different approach was needed. This was achieved by introducing human complement regulatory proteins, like CD46 and CD55 into the pig's genome. CD46 is a type 1 membrane glycoprotein expressed on most nucleated cells, that serves as a cofactor for the plasma serine protease factor I-mediated cleavage of the C3 and C5 convertase enzyme subunits C4b and C3b. Therefore, it protects the cells against complement mediated injury (DIAMOND et al., 2001). After transplantation of CD46 expressing hearts into baboons, hyperacute rejection did not occur and the recipient survived for up to 23 days (DIAMOND et al., 2001). CD55 is also known as decay accelerating factor, which increases the dissociation of the C3 and C5 convertases, also downregulating complement reaction. In pig to cynomolgus monkey kidney and heart transplantation experiments the expression of CD55 completely prevented a hyperacute rejection of the kidneys and partially of the hearts (WATERWORTH et al., 1998; ZAIDI et al., 1998).

6.3. Regulators of coagulation

Also, coagulopathy plays a big role in xenograft failure. This partly stems from inflammation but also from incompatibility of the human and the porcine coagulation regulation. Severe consequences can be bleeding, thrombocytopenia and microangiopathy causing ischemia. Factors of this reaction are CD39, the tissue factor inhibitor pathway, thrombin-thrombomodulin (TBM) interaction and the regulation of protein C (SINGH et al., 2022). Human thrombomodulin expressed on the endothelial cells binds thrombin reducing its activity. With the help of an endothelial protein C receptor (EPCR) it also converts protein C to its activated form, which inhibits factors V, VII and thrombin. Lastly it activates enzymes to dissociate residues of fibrin in the plasma (SINGH et al., 2022). Introducing this gene in combination with human CD46 and αGal knockout led to the longest lasting heterotopic abdominal xenotransplantation experiment of a pig heart in a baboon with a survival of 945 days (MOHIUDDIN et al., 2016).

6.4. Suppression of inflammatory response

Suppressing inflammatory response is another key factor in xenotransplantation. Inflammation is believed to contribute to the complement activation and coagulopathy. In the first successful pig to human transplantation, the inflammation was downregulated by human CD47 and heme oxygenase 1 (SINGIREDDY et al.,

2023). CD47 downregulates macrophage activity be binding SIRPα and inhibits phagocytosis by serving as a “don’t eat me” signal (REICHART et al., 2021; SINGIREDDY et al., 2023). Heme oxygenase 1 (HMOX1) is expressed after reperfusion injury, degrading free heme. Besides being pro inflammatory, free heme can also activate the innate immune system through MCP1 (REICHART et al., 2021; SINGIREDDY et al., 2023).

6.5. Graft overgrowth

Another problem, that presented in orthotopic pig heart to baboon transplantation experiments was graft overgrowth (DENNER et al., 2018; LANGIN et al., 2018). In an experiment in Munich, 3 baboons lived for 18, 27 and 40 days after orthotropic transplantation with the hearts of a genetically modified pigs, that had a domestic pig breed as genetic background. Echocardiography showed increased left ventricular mass, left ventricular stiffening, and decreased left ventricular filling volumes. Troponin levels remained normal. There was some infiltration of immune cells and microcoagulation but no signs of a humoral reaction (LANGIN et al., 2018). Other xenotransplantation studies have shown similar results. Therefore, it is believed, that the xenograft will continue growing in the recipient until it reaches the designated size it would have in a mature pig (LANGIN et al., 2018). This can be tackled by using smaller pig breeds, with organ weights more similar to humans. Also reducing blood pressure, early weening of cortisol and the use of the m-TOR inhibitor temsirolimus can prevent xenograft overgrowth (LANGIN et al., 2018). Lastly a knockout of the growth hormone receptor gene (*GHR*-KO) creates pigs approximately 50% smaller. Their organ size is reduced even more (HINRICHS et al., 2018). This modification was used in the pig to human xenotransplantation in 2022 (GRIFFITH et al., 2022). However, this alteration leads to multiple physiological changes in the donor pigs, such as juvenile hypoglycemia (HINRICHS et al., 2021), pronounced obesity (HINRICHS et al., 2018), alterations of multiple metabolic pathways of the liver (RIEDEL et al., 2020) and structural, proteomic and functional changes in the pituitary gland (SHASHIKADZE et al., 2023).

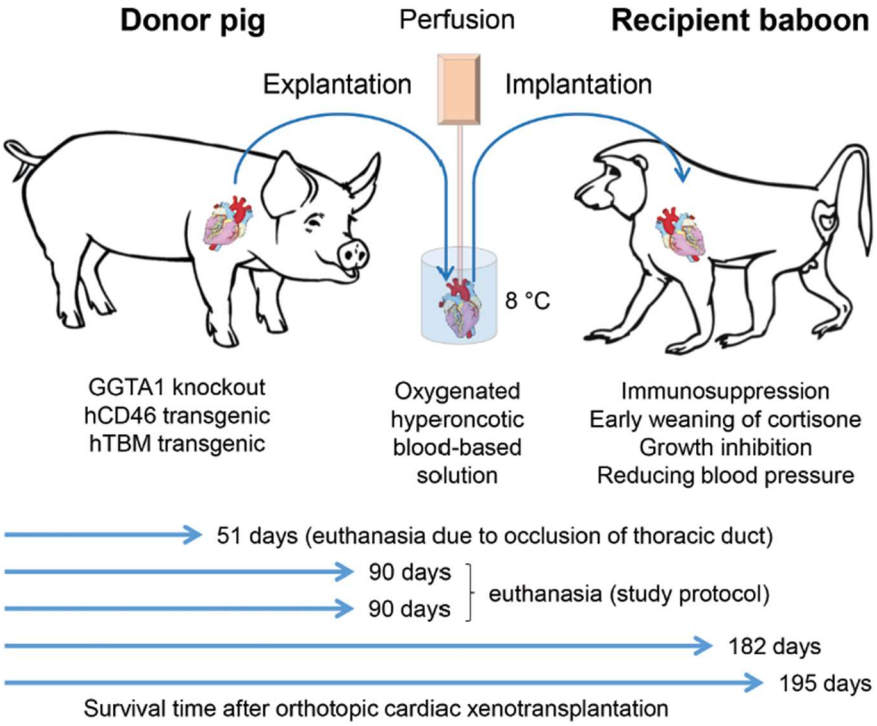


Figure 2: Principle of pig to baboon heart xenotransplantation (WOLF et al., 2019)

6.6. Number of modifications

The first multi-modified pig heart transplanted into a human had 10 genetic modifications: knockouts of *GGTA1*, *CMAH*, *B4GALNT2*/*B4GALNT2L* and *GHR*; transgenes for human CD46, CD55, TBM, EPCR, CD47 and HMOX1 (GRIFFITH et al., 2022). But it is postulated that lesser modifications might have a better outcome and are easier for the regulatory authorities to approve. Also, the breeding strategy becomes more complex with multiple loci and interactions between the transgenes are harder to rule out, arguing for source pigs with the minimum number of essential genetic modifications (KEMTER et al., 2020; REICHART et al., 2021). An overview of all genetic modifications used in pig to baboon xenotransplantation carried out at LMU is shown in Figure 2.

7. Microbiological safety

Patients receiving a transplant need to be immunosuppressed, making them especially susceptible to infection. In allotransplantation this happens quite often with HIV, Hepatitis C virus, and human cytomegalovirus, and occasionally with rabies virus (NELLORE & FISHMAN, 2018). Organs of hepatitis C patients are even used on purpose, as better therapeutic tools are available (HUCKABY et al.,

2021). Since pigs can be held under specific pathogen free conditions and screening tests can be carried out prior to the transplantation, they are appear to be a safe choice concerning infection through the xenograft (NELLORE & FISHMAN, 2018). The goal is to eliminate all pathogenic microorganisms, that are either harmful to the pig, could damage the transplant or infect the human recipient. So far screening strategies have been established for up to 100 microorganisms and clear descriptions of designated pathogen free animals and microbiological standards are defined (REICHART et al., 2021). Since bacteria, parasites and fungi can be treated in most cases, the main focus for elimination strategies lies on viruses (REICHART et al., 2021).

7.1. PCMV

Porcine cytomegalovirus (PCMV) is a roseolovirus, closely related to the human herpesviruses 6A, 6B and 7. Analyses of pig to baboon xenotransplants with relatively short survival times (4-40 days) showed PCMV infection and expressing cells in all organs of the recipient. The mechanism reducing transplant survival is still unclear (DENNER et al., 2020). However, recipient baboons showed increased levels of IL-6 and TNFα. There was no sign of αGal antibodies or humoral reaction to explain the graft failure though. An ELISA of the tissue plasminogen activator and plasminogen activator inhibitor 1 complexes (tPA-PAI-1) showed high levels of complexes. This suggests a hypercoagulable state and complete loss of the pro-fibrinolytic properties in the endothelium (DENNER et al., 2020).

7.2. Hepatitis E

Another zoonotic pathogen is the Hepatitis E virus 3 (HEV-3). It is widely distributed in wild boars and domestic pigs. Infection occurs after consumption of undercooked meat, through direct contact with infected animals or through blood transfusion. Healthy humans usually have a subclinical infection, which does not progress to acute liver failure or chronic hepatitis (DENNER, 2022). In immunocompromised people the infection progresses into chronic states. A vaccine is available in China and specific antivirals are not available at the moment (DENNER, 2022).

7.3. PERV

Porcine endogenous retroviruses (PERV) are gammaretroviruses and are integrated in the genome in up to 60 copies depending on pig breed. PERV-A and PERV-B

are polytrophic and infecting cells of different species including human cells. PERV-C is ecotrophic, infecting only pig cells. PERVs have been claimed to be able to actively infect cells in the pig, as there are different copy numbers in different tissues (DENNER, 2016). Also, recombinants of PERV-A and C have been found, which are characterized by a high replication rate and the ability to infect human cells. These are especially active in minipig lines (DENNER & SCHUURMAN, 2021). Even though the replication can be shown in vitro, transmission could not be observed in the pig to baboon transplantations (DENNER et al., 2020). A vaccination of the recipient against PERV is feasible, though elimination in the porcine genome seems to be the better alternative. This can either be accomplished by CRISPR-Cas9 (NIU et al., 2017) or by using animals naturally free of PERV-C.

7.4. Porcine Lymphotropic Herpesvirus

Porcine Lymphotropic Herpesviruses -1, -2, and -3 are gammaherpesviruses with a high prevalence in pigs. They are closely related to the Epstein-Barr virus (EBV), and Kaposi sarcoma herpesvirus in humans. So far, no association to any pig disease has been described. After experimental transplantation in minipigs, it caused post transplantation lymphoproliferative disorder (PTLD), which is similar to human PTLD linked to EBV infection. Although PHLV-1 was found in genetically modified donor pigs used in preclinical xenotransplantation studies, no transmission to the recipient could be detected (DENNER, 2021).

7.5. Other viruses

Torque teno sus virus (TTSuV), Porcine Parvoviruses and Porcine Circoviruses 1,2 and 3 are pathogens relevant to conventional pig breeding. They have no zoonotic potential, but they cause diseases in the pig. For PCV-2 there are effective vaccines available to eliminate the risk of infection (REICHART et al., 2021). The other viruses can be eliminated from pig herds by effective screening methods (DENNER, 2020a).

7.6. Screening of source pigs

A comprehensive screening of the donor pig is necessary with multiple methods. PERVs pose a special challenge, as they are integrated in the genome of every pig and cannot be eliminated by classical methods. Therefore, animals free of PERV-C should be considered for xenotransplantation. In order to detect PERVs by PCR,

primers specific for the *pol* gene, which is conserved in all PERV-classes, can be used. Specific primers must be applied to classify the subtypes. Droplet digital PCR has proven to be the most reliable method to measure the copy number of PERV (DENNER, 2020a). For a HEV an indirect detection method like ELISA or Western blot is recommended, as its genomic RNA is fragile (DENNER, 2020a). For PCMV there are reliable PCR and nested PCR tests available. However, full blood doesn't appear to be a good source material for testing. Cultivation of peripheral blood mononuclear cells (PBMCs) in the presence of mitogens significantly increases the sensitivity of PCMV detection (DENNER, 2020a). For other viruses like Torque teno sus virus (TTSuV), porcine parvovirus 1, and PCV efficient PCR and RT-PCR methods are available. Except for PERV, all potentially zoonotic viruses can be eliminated by selection of virus negative animals, vaccination, treatment with antiviral drugs, early weaning, colostrum deprivation, caesarean section and embryo transfer (EGERER et al., 2018; DENNER, 2020a).

8. Heart perfusion

To ensure xenograft survival, perfusion of the heart is necessary after explanation. Experiments perfusing with crystalloid solution (HTK or Belzer's UW solution) and keeping the hearts in plastic bags with ice cold solution led to early graft failure, after 1-30 days (LANGIN et al., 2018). A protocol using 8°C oxygenated albumin-containing hyperoncotic cardioplegic solution that contained nutrition, hormones and erythrocytes was then used (STEEN et al., 2016; LANGIN et al., 2018). The hearts were continually perfused after explantation and perfused every 15 min during implantation as shown in Figure 2. No animal was lost due to perioperative cardiac xenograft dysfunction which led to survival times of 18, 27 and 40 days (LANGIN et al., 2018). Similar approaches have led to better outcome in human allotransplantation (PINNELAS & KOBASHIGAWA, 2022).

9. Strategies to avoid organ rejection

9.1. Immunosuppressive regimen

After transplantation a non-nephrotoxic immunosuppressive regimen is key to overcome organ rejection. Initial studies used conventional drugs like cyclophosphamide, cyclosporin A, mycophenolate mofetil, and corticosteroids. Experiments were carried out with anti-CD154 mAb, which was later found to be

thrombogenic in humans (REICHART et al., 2023). Therefore regimens using anti-CD40 antibodies were established, which were used in the longest reported xenograft survival in baboons (MOHIUDDIN et al., 2016; LANGIN et al., 2018) and in the first compassionate transplantation of a pig heart into a human (GRIFFITH et al., 2022). CD40 is expressed on dendritic cells, macrophages and endothelial cells, whereas CD154 is expressed on activated CD4+ T helper cells, CD8+ cytotoxic T cells, monocytes and non-activated platelets (REICHART et al., 2023). Therefore, new antibodies against CD154 without the prothrombogenic effect are under development (COOPER et al., 2021).

9.2. HLA

Human leukocyte antigen (HLA) antibodies are known to be the cause of rejection of kidney transplants since the 1950s. Different screening test have been developed like solid phase assays or lymphocyte toxicity tests. HLA, also known as Major Histocompatibility Complex (MHC), are categorized in class I (A, B, C) and class II (DR, DQ, DP) molecules all located on chromosome 6. To date, approximately 5500 class I alleles and 1600 class II alleles have been identified, making it the most polymorphic region in the human genome (CHEN et al., 2012). Class I HLA molecules are expressed on every cell surface, presenting antigenic peptides for the generation of CD8 T-cells as response during viral infections or cancer (CHEN et al., 2012; NUNODA, 2019). HLA class II molecules are predominantly expressed on B-cells, activated T-cells and antigen-presenting cells (APCs). Antibody formation occurs in naive B-cells once they are exposed to an antigen in presence of APCs or T-helper cells (NUNODA, 2019). Allograft injury by antibodies occurs predominantly by complement activation, activating the C1q. Also, Fc receptor recruitment of inflammatory cells and release of inflammatory mediators contribute to this reaction. Risk factors for endogenous antibody formation are blood transfusion, prior transplantation, pregnancy, use of homografts or ventricular assist devices prior to transplantation (NUNODA, 2019). Sensitation with the panel reactive antibody of more than 25% results in a decrease in 5-year survival from 74% to 65% (NWAKANMA et al., 2007). Hence, crossmatching recipient and donor can avoid combinations with a high risk of organ rejection.

9.3. SLA

In pigs, the equivalent to HLA are the swine leukocyte antigens (SLA). These are

hyperpolymorphic genes, with over 150 loci located on chromosome 7, 120 of which are believed to be functional. They are divided in class I (SLA-1, SLA-2, SLA-3), class II (DRB1, BQB1, DQA) and class III genes. So far at least 50 class I and 37 class II haplotypes have been identified (HAMMER et al., 2020). The role of the MHC class I is the presentation of peptides derived from intracellular glycoproteins, typically those of viruses, to the CD8+ T-cells. Primary function of the MHC class II molecules is to present peptides from extracellular pathogens to CD4 T-cells (LADOWSKI et al., 2021). SLA is genetically close to HLA (> 70% sequence identities), so cross reaction of HLA antibodies can lead to complement activation, inflammation and ultimately rejection in recipients. Hundrieser et al. showed a significant IFN-γ secretion when PBMCs carrying HLA-DRB1*01 alleles were stimulated by porcine B-cells. Further, strong T-cell proliferation was found against SLA-DRB1*06 in 70% of humans. This demonstrates the relevance of matching HLA and SLA haplotypes to avoid immune related complications in xenotransplantation (HUNDRIESER et al., 2019). Strategies to overcome this are absorption of the antibodies by immunoabsorption column purification or genetic engineering of the pig to eliminate selected sites on the SLA causing cross reactions (LADOWSKI et al., 2021). So far studies do not allow for reliable and specific recommended HLA-SLA combinations (REICHART et al., 2021). On the other hand, highly sensitized HLA recipients, that can't find a matching organ in for allotransplantation, could get a chance in xenotransplantation, as their reaction to a modified SLA might be less severe (HAMMER et al., 2020).

9.4. Blood groups

Humans are classified in the ABO blood group system while pigs have a total of 16 blood groups (SMITH et al., 2006). The ABO blood group system is determined by oligosaccharides formed by glycosyltransferases on the surface of cells, including blood cells. These are distinguished through the erythrocyte antigen A (EAA) specific alloantibodies that play a critical role in blood transfusion and transplantation medicine (CHOI et al., 2018) Antibodies against the A and B antigens are synthesized by A and B transferases, while the transferase encoded by the O Allele is not functional. EAA is also found in pigs, resulting in cross reactivity between human blood group A-antibody and porcine A antigen. In case of blood group O this cross reactivity is absent. Therefore, using pigs with the blood group O for xenotransplantation can help overcome immune reactions due to the recipient

blood type (CHOI et al., 2018). Pigs also have a gene coding for Rhesus factor (Rh) that does not appear on erythrocytes. The minor blood group antigens also do not appear to be expressed (SMOOD et al., 2019).

10. Cloning

In animal production, genetic cloning is the production of genetic copies of individual animals using means of somatic cell nuclear transfer (SCNT) (KEEFER, 2015). In farm animals this was fist achieved by the birth of dolly the sheep in 1997. For this an oocyte, arrested in metaphase II, is first enucleated using microtools. After that a somatic cell is mechanically inserted into the perivitelline space using microtools. Then the resulting couplet is aligned between two electrodes and pulsed with an electrical current, a method called electrofusion (EDWARDS et al., 2003). With the means of pore formation through the electrical current, the somatic cell nucleus is meant to get in contact with the cytoplasm of the oocyte. The resulting embryo needs to be activated to start development, which can either be done by electrical pulses or by chemical agents. It can be cultivated until formation of a blastocyst and then be transferred to a surrogate mother (EDWARDS et al., 2003). The result are offspring, that are genetically identical to the donor cell (EDWARDS et al., 2003; KEEFER, 2015). With this technique almost all farm animals, including pigs, have been cloned (KEEFER, 2015).

11. Runs of homozygosity (ROH)

The negative effect of close relatedness of parents on the fitness of the offspring has long been recognized. Matings between related animals result in homozygous stretches along the genome of the offspring, that are identical by descent. These stretches are called runs of homozygosity (ROH) and can be used to predict the degree of inbreeding in a population, when other data, like pedigrees, is not available (BOSSE et al., 2012). Although there are other factors contributing to the distribution of inbreeding like GC-content and recombination rate, the pig has a relatively heterogeneous distribution of recombination, making ROHs a good tool to study inbreeding in these animals (BOSSE et al., 2012). ROHs have a more accurate detection of recessive and rare mutations and can describe the demographic history and domestication events (JOAQUIM et al., 2019). ROHs can be estimated by using SNP chips, which are low-cost panels analyzing about 60,000

known SNPs, covering the entire porcine genome. This method has already been used to investigate the phylogenetic relatedness of 42 Chinese pig populations (HUANG et al., 2020).

12. Yucatan minipigs

Yucatan minipigs have been bred from a stock of pigs imported from the Yucatan Peninsula of Mexico. It's a slate gray, essentially hairless pig with a docile temperament. Mean weight of mature boars is 83 kg and in mature sows it is 70 kg (PANEPINTO et al., 1978). These pigs have been well established in biomedical research and already served as donors for kidney transplantation (MA et al., 2022; FIRL et al., 2023). However, their hearts are known to have ventricular septum defects (SWINDLE et al., 1990; HO et al., 1991). They show high membranous defects analogous to humans. Some also show a patent foramen ovale (SWINDLE et al., 1990). Like most minipigs, Yucatan pigs are known for a high copy number of PERV in their genome and can actively infect human 293 cells with recombinants of PERV A and C (BITTMANN et al., 2012; DENNER & SCHUURMAN, 2021).

13. Auckland Island pigs

Figure 3: Map of Auckland Island (GMBH, 2024b)

13.1. History

Auckland Island pigs are a population of feral pigs living on a subantarctic island named Auckland Island, 560 km of South Island, New Zealand. The island is 50 km long and 10-25 km wide. A map of this island with its location in relation to

New Zealand is shown in Figure 3. There are 3 reported releases of pigs on this island by European sailors, the first in October 1807 by Captain Abraham Bristow, who discovered the island one year earlier. The second documented release was under Sir James Ross in 1840 during an Antarctic Expedition and in 1842 Maitoro released further pigs on the island while transporting Maori slaves. It is believed that more releases happened by whalers or the New Zealand Government, though no records of this could be found (ROBINS et al., 2003). In 1998 the Auckland islands were made a World Heritage site, which resulted in attempts to remove all pigs from the island. The Rare Breeds Conservation Society of New Zealand captured 17 pigs on the east coast of Auckland Island and brought them to Ivercargill (ROBINS et al., 2003). Analysis of mitochondrial D-Loop DNA from a subset of these animals revealed a close relationship to European domestic pigs, rather than European wild boars or Asian pig breeds (ROBINS et al., 2003). Microsatellite analysis also revealed a relatively low level of genetic diversity compared to other pig breeds (FAN et al., 2005).

13.2. Habits

An expedition in 1972-1973 by Challies found the pigs in relatively low numbers in the high country and at the coasts of the islands. Their food mainly consists of Pleurophyllum, Stilbocarpa and Anisotome, large leaved plants indigenous to Auckland Island. They were sighted either alone or in small groups up to 5 animals. Two thirds of the pigs are black, the rest presented from white to brown with black spots. Their litter size is reported from 1-4 piglets in the wild (CHALLIES, 1975). According to the New Zealand rare breeds society, they are distinguished by their long narrow heads and snouts and straight tails. The boars reach a live weight of 70-100 kg while the sows range between 60 and 70 kg. They are more alert and shy than domestic pig breeds (SOCIETY, 2008).

13.3. Use in research

When first brought to Ivercargill, the Rare Breeds society didn’t anticipate a use in research. Later Auckland Island pigs were considered as source animals for xenotransplantation. From these pigs a DPF herd was established, that was continuously tested free of PCMV, PLH, PCV, and hepatitis E virus. Porcine endogenous retroviruses could not be eliminated and are present in quantities of 3-30 copies (GARKAVENKO et al., 2008). In this herd animals had been identified,

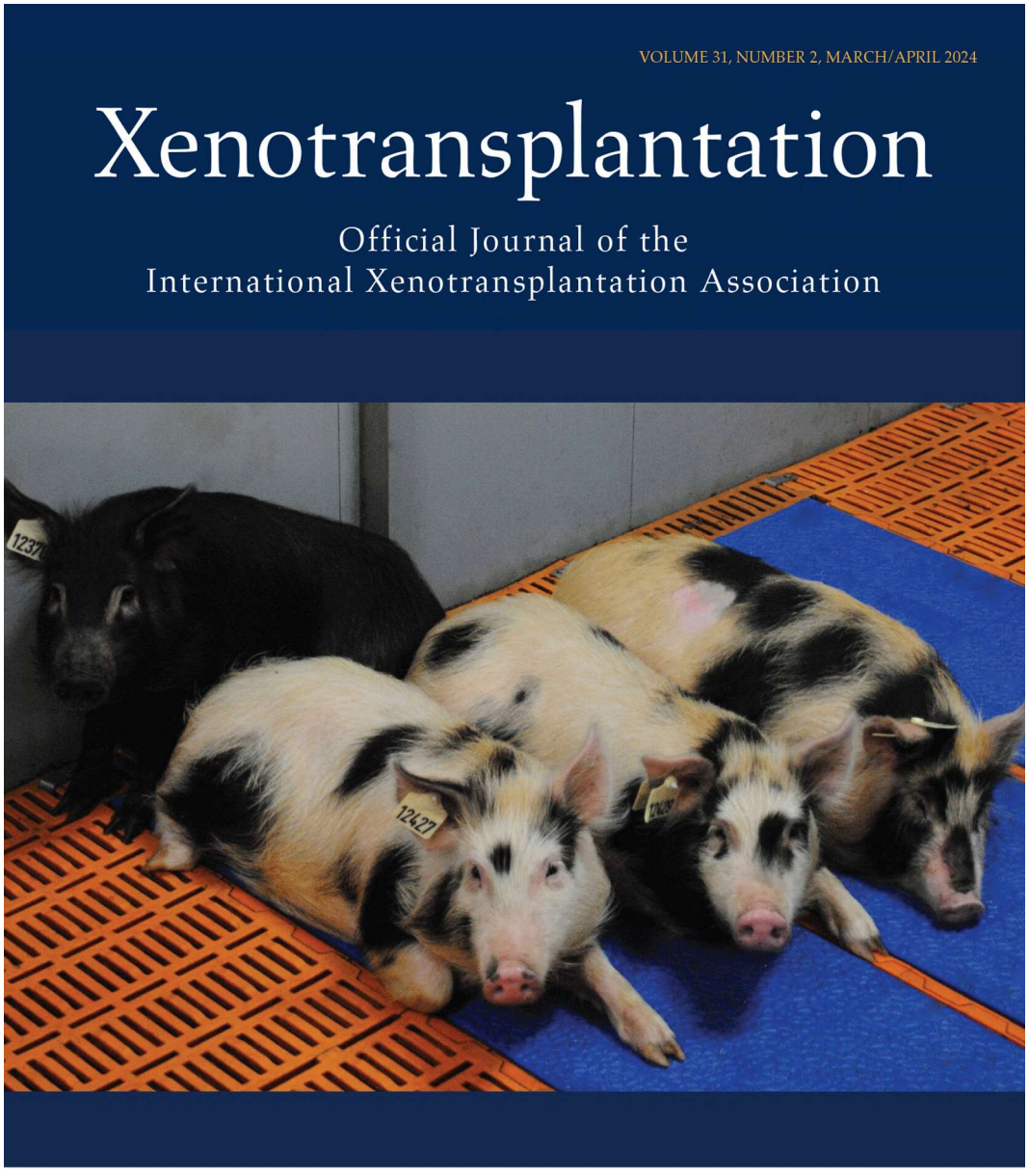
that neither infected porcine nor human cells with PERV-C. They lacked a specific full length PERV-C locus, which is present in animals capable of infecting other cells. Therefore, they were categorized as “null” for PERV transmission (GARKAVENKO et al., 2008). From 2009 onward, fourteen patients with type-1 diabetes were treated with alginate encapsulated pancreatic islets from Auckland Island pigs. No transmission of PERVs or other microorganisms was detected (WYNYARD et al., 2014).

Since Auckland Island pigs are a better size match for humans and they have an excellent hygienic status without PERV-C they appear to be the ideal donor species for xenotransplantation. Here, we analyze the genetic diversity, SLA haplotype, growth, heart size and function of a small colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich, Germany.

III. PAPER

Genetic diversity, growth and heart function of Auckland Island pigs, a potential source for organ xenotransplantation

Lange A, Medugorac I, Ali A, Kessler B, Kurome M, Zakhartchenko V, Hammer SE, Hauser A, Denner J, Dobenecker B, Wess G, Tan PLJ, Garkavenko O, Reichart B, Wolf E, Kemter E. Xenotransplantation. 2024 Mar-Apr;31(2):e12858. doi: 10.1111/xen.12858. PMID: 38646921.



Abstract

One of the prerequisites for successful organ xenotransplantation is a reasonable size match between the porcine organ and the recipient's organ to be replaced. Therefore, the selection of a suitable genetic background of source pigs is important. In this study, we investigated body and organ growth, cardiac function, and genetic diversity of a colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich. Male and female Auckland Island pig kidney cells (selected to be free of porcine endogenous retrovirus C) were imported from New Zealand, and founder animals were established by somatic cell nuclear transfer (SCNT). Morphologically, Auckland Island pigs have smaller body stature compared to many domestic pig breeds, rendering their organ dimensions well-suited for human transplantation. Furthermore, echocardiography assessments of Auckland Island pig hearts indicated normal structure and functioning across various age groups throughout the study. Single nucleotide polymorphism (SNP) analysis revealed higher runs of homozygosity (ROH) in Auckland Island pigs compared to other domestic pig breeds and demonstrated that the entire locus coding the swine leukocyte antigens (SLAs) was homozygous. Based on these findings, Auckland Island pigs represent a promising genetic background for organ xenotransplantation.

Key words Heart; Xenotransplantation; Auckland Island pigs; Genetics; Growth; PERV

Introduction

Cardiac xenotransplantation has made significant progress, demonstrating consistent long-term survival of genetically multi-modified (GM) pig hearts in both heterotopic abdominal [1] and orthotopic transplantations in baboons [2-4]. Recently, compassionate clinical xenotransplantations involving 10x GM pig hearts were performed in two patients with terminal heart diseases. The patients survived for 8 and 6 weeks, respectively [5-7]. In addition to appropriate genetic modifications to overcome xenograft rejection and coagulation dysregulation (reviewed in [8,9]), other important steps for successful cardiac xenotransplantation involve perfusion preservation of the pig hearts [10], non-nephrotoxic immunosuppression of the recipients involving T cell co-stimulation blockade ([1]; reviewed in [11]), and controlling the post-transplantation growth of the pig hearts (reviewed in [9,12]).

The size of the xenotransplant is of importance for its survival and functionality in the recipient [13]. Post-transplantation heart growth can be regulated by early weaning of glucocorticoids, lowering the recipient's blood pressure, and using the rapamycin prodrug, temsirolimus. This drug blocks the activation of the mechanistic target of rapamycin (mTOR) and prevents cardiomyocyte hypertrophy [2]. A genetic strategy to reduce the growth of the donor pigs, and subsequently their hearts, is the inactivation of the growth hormone receptor gene (*GHR*-KO) [14,15]. This modification, in conjugation with other genetic modifications, significantly prolonged the survival of orthotopic cardiac xenografts in baboons [4]. *GHR*-KO was also one of the modifications in the 10x GM donor pigs used in the already mentioned clinical compassionate use cardiac xenotransplantations [5,7]. However, *GHR*-KO may induce various physiological changes in the donor pigs, such as juvenile hypoglycemia [16], pronounced obesity [14], alterations in multiple metabolic pathways of the liver [17], and structural, proteomic, and functional changes in the anterior pituitary gland [18]. Notably, proteomic analysis of the hearts of *GHR*-KO pigs revealed only marginal changes compared to wild-type littermates [15]. Nevertheless, using a donor pig breed with organ sizes comparable to humans is preferable compared to the larger pig breeds, whose body weights and organ sizes exceed those of humans by several-fold.

GM Yucatan minipigs have been utilized for preclinical organ xenotransplantation trials [19-21], but this breed is prone to an increased occurrence of ventricular septum defects [22,23]. In addition, Yucatan minipigs are known for high expression and release of PERV [24]. An alternate option is the use of the Auckland Island pig, a breed of feral domestic pigs that originated on Auckland Island. In 1807, Captain Abraham Bristow first released these pigs on the Auckland Islands,

with at least two subsequent releases until the mid-19th century (reviewed in [25]). In 1999, the Rare Breeds Conservation Society of New Zealand removed 17 feral pigs from the main Auckland Island (**Fig. 1a**). Analysis of mitochondrial D-loop DNA from a subset of these animals suggested their European origin [26]. Wild-type Auckland Island pigs are free of numerous viruses [27] and animals maintained under designated pathogen-free (DPF) conditions already served as donors for clinical encapsulated islet xenotransplantation trials [28,29] with no indication of transmission of infectious agents to the recipients [30,31]. Due to their appropriate size for humans, they are also good candidates as donors for organ xenotransplantation. Here, we analyze the genetic diversity, SLA haplotypes, growth, and heart size and function of a small colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich, Germany.

Materials and Methods

Import of Auckland Island pigs and establishment of a breeding herd

Primary kidney cell lines were established at NZeno Limited from a male (M1) and a female (W1) piglet previously selected to be free of porcine endogenous retrovirus C (PERV-C) using standard procedures [32]. Cryopreserved cells were shipped to LMU Munich, where they were thawed and used for somatic cell nuclear transfer [33]. Cloned embryos were laparoscopically transferred to estrous synchronized recipient gilts. Pregnancy diagnosis was done by ultrasound 3 weeks after embryo transfer and subsequently at regular intervals. Animals were maintained under specific-pathogen-free (SPF) conditions in the CiMM (www.lmu.de/cimm) [34]. They were housed in groups, separated by sex, and kept under a 12-h light/dark cycle. They were fed a custom-made diet tailored to their needs (**Table S1**), designed by the Chair of Animal Nutrition and Dietetics, LMU Munich. All experiments were performed after the permission of the Government of Upper Bavaria under file name ROB-55.2-2532.Vet_02-19-195.

AO blood group and SLA genotyping

Determination of AO blood group was performed by multiplex polymerase chain reaction (PCR). Alleles of SLA class I and II genes were determined by Sanger sequencing of PCR products using cDNA of PBMCs and/or spleen and heart tissue as templates. Primers were designed first in conserved regions based on alignments of all known alleles in the Immuno-Polymorphism Database (IPD)-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/SLA/), and if indicated in allele-specific regions (primers listened in **Table S2**).

Genome-wide homozygosity analysis

To investigate the length and distribution of runs of homozygosity (ROH) in Auckland Island pigs, we performed SNP genotyping using the PorcineSNP60 v2 BeadChip, which contains 64,232 SNPs covering the pig genome almost uniformly. A total of 93 Auckland Island pigs, including two founder animals (M1 and W1 in **Fig. 1b**) and their offspring (**Fig. 1c**), were genotyped. In addition to these Auckland Island pigs, we downloaded from Yang et al. [35] the SNP genotypes of four of the world's most popular commercial breeds: Duroc, Landrace, Large-White and Pietrain. This study analyzed four Duroc, seven Landrace, two Large-White, and three Pietrain subpopulations. We pooled these and excluded animals that showed high unbiased additive genetic relatedness [36]. After iteratively excluding highly related animals (one per each highly related pair), we were left with 40 Duroc, 40 Landrace, 36 Large-White, and 25 unrelated Pietrain pigs. After the creation of “ped” and “fam” input files, ROH analyses were performed with PLINK using 47,234 SNPs informative in a global set of pig breeds. Identical parameter settings were used for Auckland Island pigs and four cosmopolitan breeds: "--homozyg-window-snp 100 --homozyg-window-het 1". To visualize the distribution of ROHs, the PLINK output file was converted into a diagram similar to a Manhattan plot, where the homozygous segments (ROH) are given the value 1 and the non-homozygous ones are given the value 0.

Growth measurements and echocardiography

Echocardiography was performed at 8, 12, 16, 22, 30, 40, and 155 weeks of age, and the animals were weighed each time before the examination. For echocardiography, animals were anesthetized using ketamine (20 mg/kg; Ursotamin®, 100 mg/ml, Serumwerke Bernburg) and azaperone (2 mg/kg; Azaporc®, 40 mg/ml, Serumwerke Bernburg) and maintained using propofol (4 mg/kg/h; Propofol 2% (20 mg/mL), MCT Fresenius). To eliminate any discomfort, meloxicam (0.4 mg/kg; Metacam 20 mg/ml, Boehringer Ingelheim) was applied. Front and rear leg height, crown-rump length, head length, thoracic circumference, and abdominal circumference were measured and pictures were taken in right lateral recumbency on top of a reference board.

Anesthetized pigs were placed on an echocardiography examination table with a hole (Eickemeyer, Germany) in right lateral recumbency. First, a 6-channel electrocardiogram (Eickemeyer Veterinär PC-EKG, Eickemeyer, Germany) was generated over 5 min and saved electronically. Then, transthoracic echocardiography was performed as described previously [37,38] using an Esaote MyLab X8 Vet ultrasound machine with a P 2-9 phased array probe for animals up

to 15 kg and a P 1-4 phased array probe for larger animals. The probe was placed underneath the pig in the right 3rd to 5th intercostal space behind the front leg. Echocardiography was performed using the standard planes defined for dogs and cats [39], slightly modified for use in pigs. Functional analyses were performed with the color flow (CF), pulse wave (PW), and continuous wave (CW) in tissue Doppler imaging. In addition, apical views were generated with the probe placed caudally in the 4th to 6th intercostal space.

Tansesophageal echocardiography was performed with an ST2612 Phased Array TEE-Probe from Esaote in animals over 25 kg. In that case, animals were kept in right lateral recumbent position. The aortic outflow tract and the mitral valve including the left atrium were shown and Doppler measurements of both valves taken.

Altogether 3 cardiac cycles were analyzed using the internal software of the Esaote MyLab X8 Vet. The left ventricular (LV) function was obtained in B-mode, long axis (LAX) LV dimensions in M-mode. LV ejection fraction (EF) and fractional shortening (FS) were calculated according to the Teichholz method [40]. Aortic valve and the left atrial measurements were done, aortic valve leaflets watched in B-mode. The dimensions of the pulmonary valve were measured in B-mode, and the flow through it was shown using- pulse wave Doppler; mitral flow and aortic flows were measured in the same way.

Blood sampling and clinical chemistry

Blood samples were collected from the jugular vein at 12, 22, 40, and 155 weeks of age. 1 mL was sampled in an EDTA vial (Sarstedt) and 5 mL was sampled in a serum vial (Sarstedt). EDTA blood was stored at 4 °C immediately after sampling for hematology analysis. Hematological parameters were measured using an Abaxis VetScan HM5. A manual differentiation of leukocytes in blood smears was performed by an experienced lab technician. For serum preparation, the blood was stored at room temperature for 20 min, and centrifuged at 1800×*g* and 4 °C for 20 min. The serum was then transferred to collection tubes and stored at 4 °C before analysis. The samples were analyzed for standard clinical chemical parameters with a Cobas 311 Analyser System (Roche Diagnostics International AG, Rotkreuz, Switzerland) and adapted test kits.

Necropsy

Necropsies were performed immediately after the last examination at 22, 40, or 155 weeks of age. Animals still under anesthesia were injected with fentanyl (7 µg/kg, Fentadon 50 µg/ml, Dechra), ketamine (Ursotamin®, 100 mg/ml, Serumwerke Bernburg), and xylazine (Xylazine 20 mg/ml, Serumwerke Bernburg). After 5 min, they were exsanguinated by cutting their carotid artery. Necropsy was followed by a standardized sampling protocol [41]. Thoracic and abdominal organs were weighed to the nearest gram. The heart size was measured at the left coronary from the base to the apex. The heart width was also measured at the left coronary artery from side to side. The heart circumference was measured at the base of the heart. Both ventricles were opened and the rest of the blood was removed to get an accurate weight. Tissue samples for histology were taken 2 cm above the apex and stored in 4% formaldehyde, 4% paraformaldehyde, or methacarn. The size and weight of the kidneys were measured. The kidneys were cut longitudinally and vertically to look for pathologies. Samples for histologic examination were taken 1 cm next to the hilus from the cortex and medulla.

Data analysis

Body weight and size data, heart rate as well as echocardiographic data were analyzed using PROC MIXED (SAS 8.2; SAS Institute Inc., Cary, NC, USA), taking the fixed effects of age, sex, and the interaction age*sex as well as the random effect of the individual animal into account. Heart weight and size data were analyzed using PROC GLM (SAS 8.2), taking the fixed effects of age, sex, and the interaction age*sex into account. Linear regressions and correlations between heart and body weights of male and female Auckland Island pigs were calculated using GraphPad Prism.

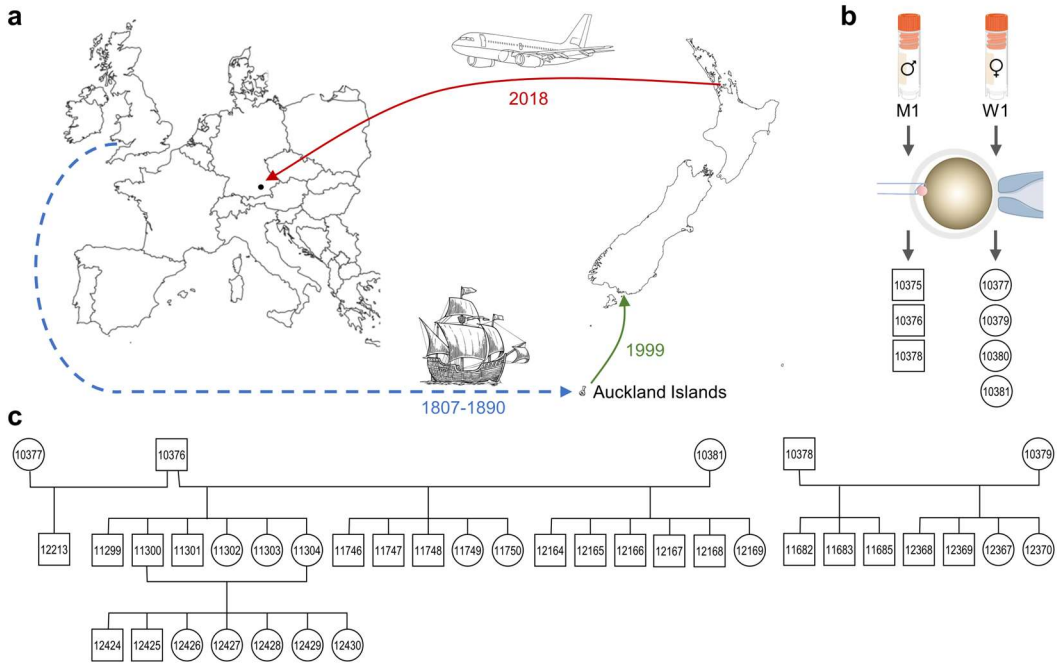


Figure 1. Establishment and genetic characterization of an Auckland Island pig breeding colony in Munich, Germany. **a)** History of the transfer of Auckland Island pigs to the Auckland Islands, from there to Invercargill, and of cells to Munich. **b)** Establishment of male and female founder pigs from a male and a female PERV-C free Auckland Island pig cell line by somatic cell nuclear transfer (SCNT). **c)** Pedigree of the animals used for phenotypic characterization.

Results

Establishment of an Auckland Island pig colony at CiMM, LMU Munich

Male and female primary kidney cell lines (M1 and W1), previously selected to be PERV-C-free, were obtained from NZeno Limited (**Fig. 1a**). A total of 373 male and female embryos, generated by SCNT, were transferred to 3 recipients. Two of the recipients became pregnant and gave birth to a total of 3 male and 4 female piglets (**Fig. 1b**). All of the 7 liveborn piglets could be raised to adulthood. Two boars (#10376, #10378) and 3 sows (#10377, #10379, #10381) served as founders of the breeding colony at CiMM, LMU Munich (**Fig. 1c**). These founder pigs were naturally mated to produce F1 generation, and later an F2 generation was also produced. Two founder pigs, 25 F1 offspring (17 males, 8 females), and 7 F2 offspring (2 males, 5 females) were used for phenotypic analysis (**Fig. 1c**), and a higher number of animals was used for SNP analysis.

Growth characteristics of Auckland Island pigs in Munich

The body dimensions of Auckland Island pigs were regularly measured as shown in **Fig. 2a**. The height at withers increased from approximately 30 cm at 8 weeks to approximately 70 cm at 155 weeks of age. From the age of 40 weeks, females were significantly smaller than males (**Fig. 2b**). The body weight of both male and female Auckland Island pigs increased from 3 kg to >100 kg within the same period, with males having greater body weight than females (**Fig. 2c**). Additional parameters of body growth are presented in **Table S3**.

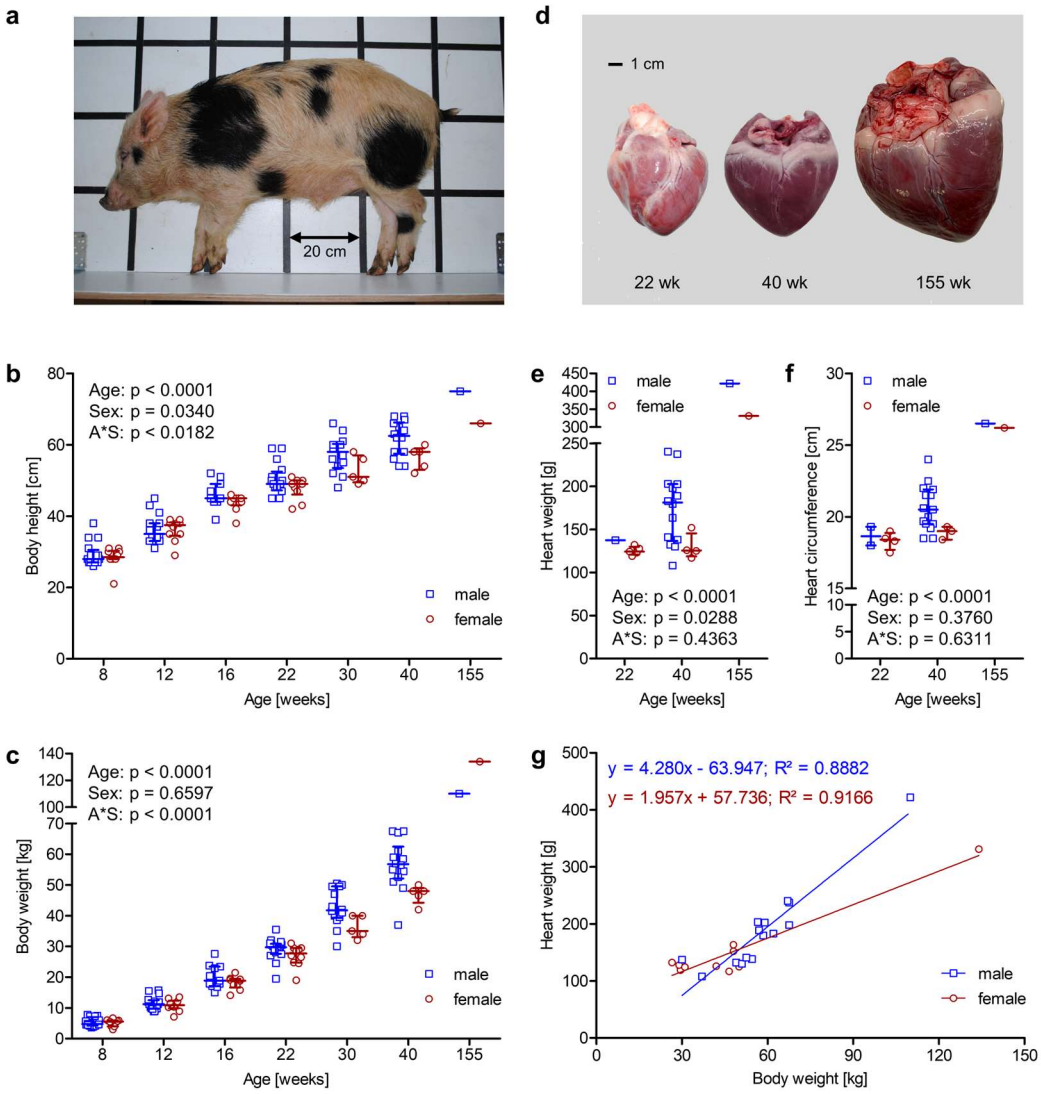


Figure 2. Body and cardiac growth of Auckland Island pigs in Munich. **a)** Grid for the measurement of body dimensions. **b)** Body height. **c)** Body weight. **d)** Representative hearts at different ages. **e)** Heart weight. **f)** Heart circumference. **g)** Regression between body weight and heart weight. Significant effects of Age, Sex and the interaction Age*Sex (A*S) are indicated.

Representative hearts at 20, 40, and 155 weeks of age are shown in **Fig. 2d**. The heart weights ranged between 119 to 137 g in the 20-week group and between 108 to 240 g in the 40-week group. The hearts of the two 155-week-old animals weighed 331 and 422 g. The corresponding data for heart circumference are shown in **Fig. 2e**. The age-related increase in heart weight was correlated with body weight, with a trend of higher relative heart weights in males than in females (**Fig. 2f**). Histologically, the hearts were free of any pathologies.

The weights of hearts, kidneys and other organs are presented in **Table S4**. Macroscopically, the kidneys displayed a good ratio of cortex to medulla and no cysts or signs of dilation. No histological changes were observed. Of all the animals investigated, there was one case of pericarditis of unknown origin. Another animal had liver cirrhosis, whereas its littermates, living under the same housing conditions, showed no alterations in the liver.

Cardiac function of Auckland Island pigs in Munich

Cardiac dimensions and functional parameters were determined by echocardiography (**Fig. 3a**). Cardiac parameters of different age groups are summarized in **Table S5**. Reference values were determined by using the 95% confidence interval (CI) [42]. Size-dependent parameters, such as left ventricular inner diameter in diastole (**Fig. 3b**) and aortic diameter (**Fig. 3c**) increased with age, with a trend of a smaller aortic diameter in females than in males. The mean left ventricular ejection fraction (LVEF) increased from 58% at age 8 weeks to 70% in the 40-week group, with a trend of a smaller LVEF in females than males of the younger age group (**Fig. 3d**). Other functional parameters, such as flow parameters of all valves, E and A wave, isovolumic contraction time (IVCT) and isovolumic relaxation time (IVRT) remained constant among the different age groups (**Table S5**). In some animals, trivial, hemodynamically insignificant tricuspid regurgitations were seen. However, no visible morphologic alterations were observed, especially no incompetencies.

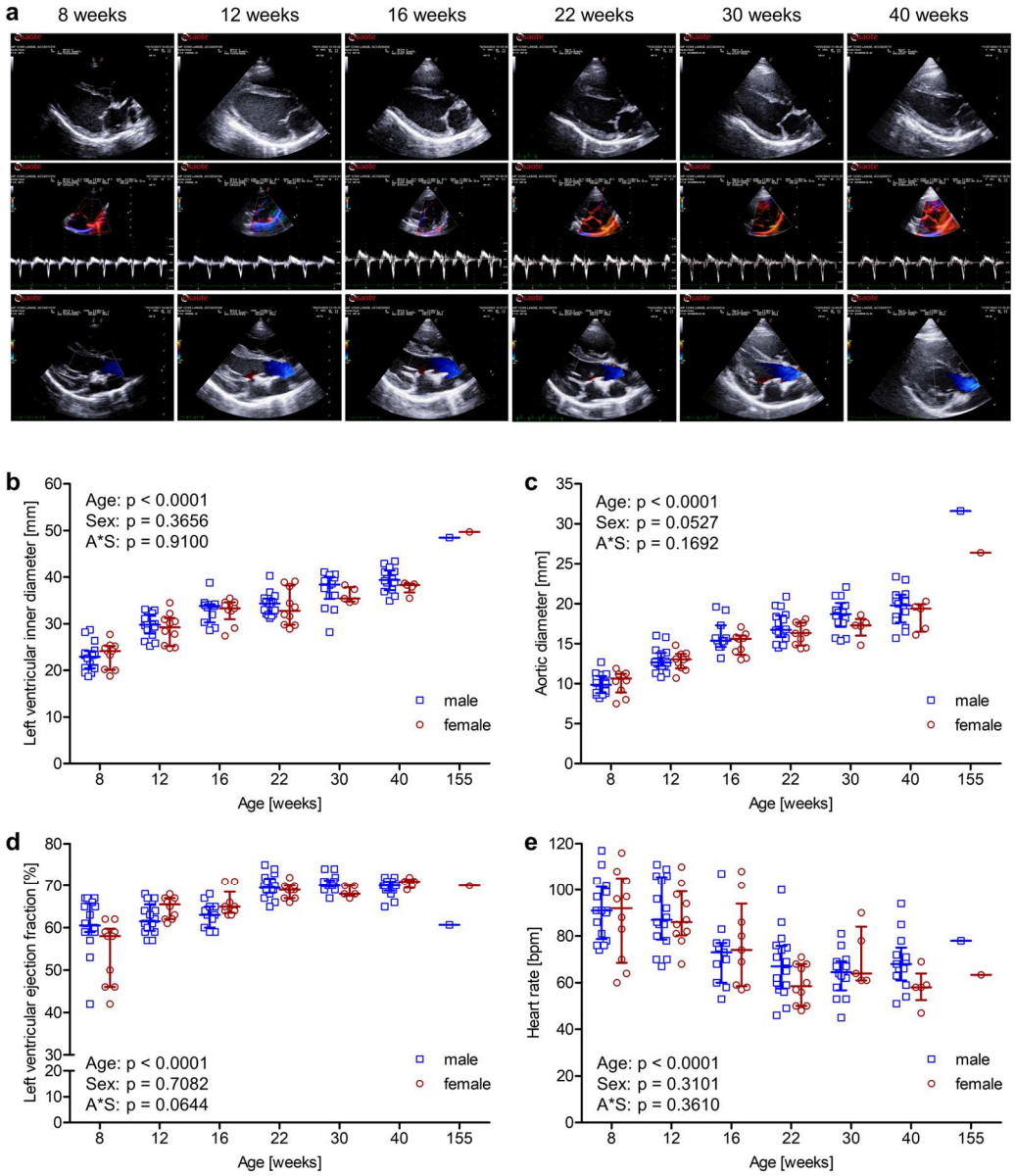


Figure 3. Echocardiography of Auckland Island pigs in Munich. **a)** Representative longitudinal echocardiography images of animal #12369 of right parasternal 4 chamber long axis view (upper panel), tissue Doppler images of lateral mitral valve at apical view (middle panel), and color flow Doppler images of the aortic valve in long axis view (lower panel) at different ages as indicated. **b)** Left ventricular inner diameter. **c)** Aortic diameter. **d)** Left ventricular ejection fraction. **e)** Heart rate. Significant effects of Age, Sex and the interaction Age*Sex (A*S) are indicated.

Homozygosity analysis of the Auckland Island pig colony at CiMM

A total of 93 Auckland Island pig DNA samples, including the cell lines M1 and W1, two founder animals (#10375 und #10379) and their offspring, were SNP genotyped. The SNP genotyping data from Auckland Island pigs was also compared to the above-described 47,234 SNPs identified in the global dataset from 4 pig breeds to estimate the expected heterozygosity and runs of homozygosity across five breeds. The total length of ROH (Mb) across the autosomal genome and their genome proportion was estimated for each animal. ROH analysis for the M1 and W1 cell lines and some chosen offspring including the ones with the lowest and highest proportion of genome in ROHs are visualized in **Fig. 4a.** The two relatively long chromosomes 6 (170.8 Mb) and 13 (208.1 Mb) show only relatively short ROHs in the female (W1) and no ROHs in the male (M1) cell line. Consequently, chromosomes 6 and 13 in our Auckland Island pig colony have a very low proportion of ROHs, 4.4 %, and 6.2 %, respectively. On the other hand, the two relatively short chromosomes 17 (63.5 Mb) and 18 (55.9 Mb) have very high proportions of ROHs (94.0 % and 64.5 %, respectively) in our Auckland Island pig colony in Munich.

The simple statistics of ROHs across the breeds are shown in **Table 1**. Auckland Island pigs clearly show the lowest heterozygosity (0.123), followed by Duroc (0.281), Pietrain (0.320), Large-White (0.331), and Landrace (0.336). In the 93 Auckland Island pigs analyzed here, identical haplotypes are inherited from both parents and thus form long tracts of homozygous genotypes, which on average add up to 626.2 Mb (27.6 %) over the entire genome (**Table 1**). This is 2.8-4.4 times higher than for the four intensively selected and economically most important pig breeds. Other ROH summary statistics such as the number of ROHs per animal and the number of SNP per ROH (**Table 1**) also clearly confirm that our Auckland Island pig colony is many times more homogeneous than long-term and intensively selected commercial breeds.

Uniformity of AO blood group and SLA genotype of Munich Auckland Island pigs

The AO blood group of Auckland Island pigs is uniformly O within the breeding herd. The SNP analysis revealed that all Munich Auckland Island pigs are also uniform in their SLAs. Of note, ROHs of SNPs were present between SSC7 19.5-30.8 Mb encompassing the 2.6 Mb SLA gene clusters region. The constellation of SLA class I and SLA class II genes are shown in **Fig. 4b**.

Table 1. The simple statistics of runs of homozygous (ROH) across Auckland Island pigs and the four most popular commercial breeds worldwide. Maximal values are in bold.

	Auckland Island	**Duroc**	**Landrace**	**Large-White**	**Pietrai**
Number of animals	93	40	40	36	25
Average number of ROHs per animal (±SD)	**38.3 (±4.2)**	25.8 (±6.1)	16.0 (±**6.6**)	16.9 (±5.5)	18.2 (±5.5)
Range (Minimum-Maximum)	***30 - 48***	*16 - 46*	*1 - 32*	*7 - 32*	*10 - 28*
Average sum of all ROHs per animal (in Mb) (±SD)	**626.2 (±92.6)**	226.4 (±62.5)	144.0 (±76.6)	150.2 (±72.5)	165.7 (±42.9
Range (Minimum-Maximum)	***427.9 - 844.1***	*107.0 - 384.7*	*13.5 - 346.7*	*44.8 - 384.5*	*80.7 - 248.0*
Number of SNP in ROHs per Animal (±SD)	**15,299 (±2,171)**	5,485 (±1,496)	3,506 (±1,839)	3,665 (±1,729)	4,013 (±1,017
Range (Minimum-Maximum)	***10,331 - 20,784***	*2,727 - 9,503*	*325 - 8,190*	*1,055 – 9,007*	*1,908 - 5,829*
Proportion of genome in ROHs per animal (±SD)	**27.6 (±4.1)**	10.0 (±2.8)	6.4 (±3.4)	6.6 (±3.2)	7.3 (±2.3)
Range (Minimum-Maximum)	***18.9 -37.3***	*4.7 -17.0*	*0.6 -15.3*	*2.0 -17.0*	*3.6-11.*

Clinical-chemical reference values of Auckland Island pigs in Munich

The clinical-chemical reference values of all groups are presented in **Table S6**. Compared to reference values of German Landrace pigs [43], Auckland Island pigs have higher creatinine kinase, hemoglobin, and hematocrit values, but lower leukocyte counts with up to 54% lymphocytes.

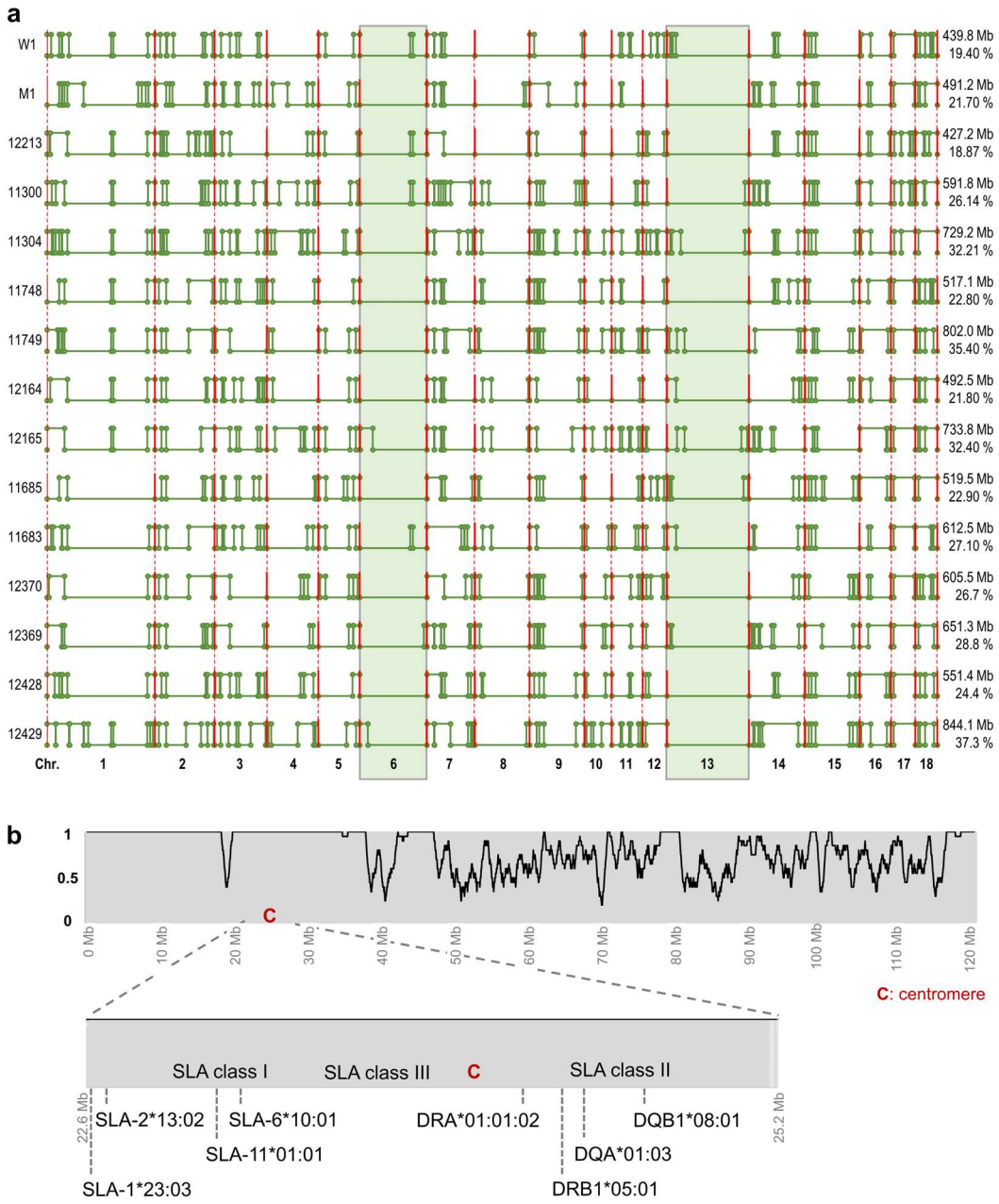


Figure 4. a) Runs of homozygosity (ROH) analysis of the initial cell lines, the founder animals, and their offspring. A value of 0 indicates heterozygous genome segments and a value of 1 indicates homozygous segments. The chromosomes are separated by red lines, the chromosome numbers are indicated below the graph. Left site upper number: sum of all ROHs per animal (in Mb); lower number: proportion of genome in ROHs per animal. **b)** Region of homozygosity of SNP haplotype of male and female Auckland Island pig on SSC7 harboring the MHC/SLA locus. Haplotype identification for SLA class I alleles was done from cDNA of PBMC, for SLA class II from cDNA of PBMC and tissue.

Discussion

Xenotransplantation of solid organs from pigs to humans is being explored as an alternative to human organ allotransplantation, aiming to alleviate the critical issue of transplantable human organ shortages. Substantial research has been done to overcome key challenges in organ xenotransplantation, including immune rejection due to molecular incompatibilities across species, variations in organ sizes between pigs and humans, and the potential transmission of pathogens. A widely adopted approach to tackle these challenges is the creation and utilization of genetically modified (GM) pigs for xenotransplantation. Identifying an optimal donor pig breed for organ xenotransplantation has proven difficult. In this current study, we propose that Auckland Island pigs may be a superior option for pig-to-human organ xenotransplantation.

Utilizing male and female Auckland Island pig primary kidney cell lines, we developed a breeding colony of Auckland Island pigs at LMU Munich through SCNT. All founder animals resulting from the initial SCNT (comprising 3 males and 4 females) exhibited good health and displayed no cloning artifacts, affirming the cloning efficiency of this highly inbred pig breed. By subsequent breeding of the founder animals, we successfully established F1 and F2 progenies and raised them to adulthood without encountering any significant health issues. We assessed the viability of these Auckland Island pigs as potential organ donors for xenotransplantation. This involved a comprehensive analysis of their body and organ sizes, cardiac function, genetic homozygosity, pathogen level, and the presence of SLA haplotypes.

The primary objective of this current study was to delineate the growth and cardiac function of Auckland Island pigs in Munich and to assess their suitability for human transplantations. In adult humans, a normal heart weighs from 156 to 422 g in women and from 188 to 575 g in men [44]. Notably, larger domestic pig breeds, with adult weights reaching up to 350 kg, have hearts that are too big for human transplantation. To ensure the appropriateness of size, Längin et al. utilized the hearts of juvenile cross-bred pigs aged 6-12 weeks (a cross between German Landrace and Large White) for transplantation in non-human primates [45]. However, these hearts exhibited post-transplantation growth in preclinical trials, leading to graft failure within 28 days [45]. This data suggests that, even when isolated at an early age, porcine organs retain the capacity to grow in accordance with the specific pig breed. An alternative strategy to address organ size discrepancies involved knocking out the growth hormone receptor (*GHR*) in domestic pig breeds, resulting in smaller bodies and organ sizes suitable for human transplantation [14,15].

Nevertheless, *GHR*-KO can lead to various pathophysiological complications in donor pigs [14,16-18], restricting the long-term viability of this approach. In another approach to address the size mismatch issues, GM Yucatan minipigs were used in preclinical organ xenotransplantation trials [19-21]. However, Yucatan pigs were reported to exhibit a high occurrence of ventricular septum defects [22,23]. Therefore, finding a donor pig breed with organ sizes comparable to those of humans and devoid of functional defects in the organs remains a crucial objective. In the wild, adult Auckland Island boars were reported to weigh an average of 41.7 kg while adult sows weigh about 37.3 kg [25]. On the other hand, provided with a regular diet, adult Auckland Island pigs bred in Munich are similar to those bred by NZeno Limited in New Zealand where they weigh 100-140 kg for adult males and 65-104 kg for adult sows (unpublished communication). Based on our findings, the hearts of adult Auckland Island pigs aged 9-12 months demonstrate an optimal weight and size for potential transplantation into adult humans (**Fig. 2e, f**).

Notably, no additional genetic modifications or pharmacological interventions are deemed necessary to regulate heart growth post-transplantation in humans or non-human primates. Furthermore, throughout the study, Auckland Island pig hearts displayed normal structure and functionality across various age groups, with no structural or functional anomalies observed. Additionally, we can predict the heart weight very well using 2D transthoracic echocardiography. Based on these findings, we propose that an Auckland Island pig heart is a superior option for xenotransplantation into humans, and a sized-matched heart obtained from an adult Auckland Island pig is anticipated to maintain its size post-transplantation. Additionally, we have presented reference data on the functional aspects of the Auckland Island pig heart. This information will be invaluable in screening potential Auckland Island donor pigs, identifying those with cardiac abnormalities, and facilitating their inclusion in pre-clinical or clinical trials.

Cross-species transmission of potentially zoonotic viruses during pig-to-human xenotransplantation poses a significant concern. In the first pig-to-human heart transplantation, the porcine cytomegalovirus, a porcine roseolovirus (PCMV/PRV) DNA was detected in the recipient, possibly contributing to the patient's pathological condition [5]. Preclinical trials have indicated that PCMV/PRV can be transmitted to the host, leading to a reduction in xenograft survival time [46,47]. However, early weaning has been shown to eliminate PCMV/PRV [34]. In contrast, porcine endogenous retroviruses (PERVs) are integrated into the pig genome and cannot be eliminated by conventional methods [48,49]. While PERVs can infect human cells *in vitro* [50], there have been no reported *in vivo* infections of human

cells by PERVs in xenotransplantation clinical trials [47]. PERV-A and -B can infect both human and pig cells, while PERV-C infects only pig cells [51,52]. Recombination between PERV-A and PERV-C can generate PERV-AC which is 500-fold more infective than the parental PERV-A [53]. Therefore, using PERV-C-free animals is crucial to reduce the risk of PERV infection [47], as mandated by the Food and Drug Administration (FDA) for xenotransplantation. Various strategies have been implemented to minimize the risk of PERV transmission, including using pig breeds with low or no PERV expression, employing antiretroviral drugs to prevent PERV infection, and utilizing donor pigs with PERV gene knockouts [47]. Notably, Auckland Island pigs are free from a wide range of porcine pathogens, including PERV-C [47]. In clinical trials of Auckland Island pig islet xenotransplantation, no PERVs were transmitted to the human recipients [30,31,47], making them preferable tissue and organ donors for xenotransplantation.

The founder animals and their offspring exhibited high ROH levels, indicating a high degree of inbreeding. Surprisingly, we did not find any signs of inbreeding depression. This resilience might be attributed to a gradual rise in genomic homozygosity and the elimination of deleterious alleles through natural selection [54]. Notable exceptions were chromosomes 6 and 13, which displayed considerably lower ROH levels compared to other chromosomes. Interestingly, these chromosomes harbor genes associated with the regulation of meat quality traits [55] and characteristics such as litter size and uterine horn length [56], respectively. A pivotal genomic region is found on chromosome 7, housing the major histocompatibility complex (MHC) or swine leukocyte antigen complex (SLA) [57]. SLAs are analogous to human leukocyte antigens (HLAs) and play a crucial role in the immune system. Pregnant women, individuals who have undergone blood transfusions, and transplant recipients develop antibodies against HLAs [58]. In such HLA-sensitized human recipients, anti-HLA antibodies may cross-react with SLAs on the xenograft, resulting in graft failure [59]. This underscores the significance of SLAs in the context of xenotransplantation.

Removal of SLAs from donor pigs has been considered to avoid immune rejection of the xenograft [60,61]. However, SLA deletion can render the pig at risk for infectious complications [62]. Alternatively, site-specific SLA mutations are recommended to avoid the reactivity with anti-HLA antibodies [63]. Moreover, the careful detection of anti-SLA antibodies in the xenograft recipients can assist in reducing cross-reactivity issues. Implementing these strategies requires a precise classification of SLAs in the donor pig breed. SLAs are hyper-polymorphic genes, with over 150 loci encoded in the SLA region at chromosome 7, at least 120 of

which are believed to be functional [63]. SLAs are categorized into SLA class I (SLA-1, SLA-2, SLA-3), class II (DRB1, DQB1, DQA), and class III molecules. To date, at least 50 class I and 37 class II SLA haplotypes have been identified [64], and the database continues to expand. Intriguingly, in our Auckland Island pig colony in Munich, the entire SLA region on chromosome 7 was found to be homozygous in all animals. Although MHC haplotypes could be determined for numerous SLA genes, it failed for some, such as SLA-3. This limitation might be attributed to preliminary pig genome annotations that are currently under revision for the chromosomal SLA gene complex (Sabine E. Hammer, personal communication). Nevertheless, the homozygosity of SLAs in our Auckland Island pigs simplifies potential strategies to overcome SLA-mediated xenograft rejection in preclinical and clinical trials. The blood group of the donor pig is also an important consideration in xenotransplantation trials. Pigs with blood type O are preferred, as they are less likely to elicit a strong immune response in humans, particularly if the recipient's blood type is not O [65-67]. In addition to homozygous SLAs, all Auckland Island pigs in Munich possess the blood group O, reinforcing the notion that these pigs may be a superior organ source for xenotransplantation.

Conclusion

Auckland Island pigs exhibit excellent cardiac function, and their heart size closely resembles that of humans. No pathologic alterations were observed in echocardiography, necropsy, or histologic assessments. Since Auckland Island pigs in Munich are free of PERV-C and are kept under designated pathogen-free (DPF) conditions, the risk of infection through xenograft is minimized. Moreover, Auckland Island pigs are homozygous for SLAs which simplifies further strategies to overcome rejection. They possess blood group O, naturally addressing a crucial barrier in xenotransplantation. With minimal genetic modifications, such as the elimination of carbohydrate xeno-antigens, and the expression of human complement pathway regulatory proteins and human thrombomodulin, Auckland Island pigs emerge as a promising organ source for xenotransplantation.

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Conflict of Interest Statement

P.L.J.T. and O.G. are founders of NZeno Limited, Auckland, New Zealand; B.R., E.W., and E.K. are founders of XTransplant GmbH, Starnberg, Germany.

References

1. Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun.* 2016;7:11138.
2. Längin M, Mayr T, Reichart B, et al. Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature.* 2018;564(7736):430-433.
3. Reichart B, Längin M, Radan J, et al. Pig-to-non-human primate heart transplantation: The final step toward clinical xenotransplantation? *J Heart Lung Transplant.* 2020;39(8):751-757.
4. Mohiuddin MM, Goerlich CE, Singh AK, et al. Progressive genetic modifications of porcine cardiac xenografts extend survival to 9 months. *Xenotransplantation.* 2022;29(3):e12744.
5. Griffith BP, Goerlich CE, Singh AK, et al. Genetically Modified Porcine-to-Human Cardiac Xenotransplantation. *N Engl J Med.* 2022;387(1):35-44.
6. Mohiuddin MM, Singh AK, Scobie L, et al. Graft dysfunction in compassionate use of genetically engineered pig-to-human cardiac xenotransplantation: a case report. *Lancet.* 2023;402(10399):397-410.
7. In Memoriam: Xenotransplant Patient Lawrence Faucette. 2023; https://www.umms.org/ummc/news/2023/announcing-the-passing-of-lawrence-faucette. Accessed 30.12.2023, 2023.
8. Kemter E, Schnieke A, Fischer K, Cowan PJ, Wolf E. Xeno-organ donor pigs with multiple genetic modifications - the more the better? *Curr Opin Genet Dev.* 2020;64:60-65.
9. Reichart B, Cooper DKC, Längin M, Tönjes RR, Pierson RN, Wolf E. Cardiac xenotransplantation: from concept to clinic. *Cardiovasc Res.* 2023;118(18):3499-3516.
10. Langin M, Reichart B, Steen S, et al. Cold non-ischemic heart preservation with continuous perfusion prevents early graft failure in orthotopic pig-to-baboon xenotransplantation. *Xenotransplantation.* 2021;28(1):e12636.
11. Singh AK, Goerlich CE, Zhang T, Lewis BGT, Hershfeld A, Mohiuddin MM. CD40-CD40L Blockade: Update on Novel Investigational Therapeutics for Transplantation. *Transplantation.* 2023;107(7):1472-1481.
12. Reichart B, Längin M, Denner J, Schwinzer R, Cowan PJ, Wolf E. Pathways to Clinical Cardiac Xenotransplantation. *Transplantation.* 2021;105(9):1930-1943.
13. Denner J, Reichart B, Längin M. Does size matter? *Xenotransplantation.* 2018;25(2):e12383.
14. Hinrichs A, Kessler B, Kurome M, et al. Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver. *Mol Metab.* 2018;11:113-128.

15. Hinrichs A, Riedel EO, Klymiuk N, et al. Growth hormone receptor knockout to reduce the size of donor pigs for preclinical xenotransplantation studies. *Xenotransplantation.* 2021;28(2):e12664.
16. Hinrichs A, Renner S, Bidlingmaier M, Kopchick JJ, Wolf E. MECHANISMS IN ENDOCRINOLOGY: Transient juvenile hypoglycemia in growth hormone receptor deficiency - mechanistic insights from Laron syndrome and tailored animal models. *Eur J Endocrinol.* 2021;185(2):R35-r47.
17. Riedel EO, Hinrichs A, Kemter E, et al. Functional changes of the liver in the absence of growth hormone (GH) action - Proteomic and metabolomic insights from a GH receptor deficient pig model. *Mol Metab.* 2020;36:100978.
18. Shashikadze B, Franzmeier S, Hofmann I, et al. Structural and proteomic repercussions of growth hormone receptor deficiency on the pituitary gland: Lessons from a translational pig model. *J Neuroendocrinol.* 2023:e13277.
19. Ma D, Hirose T, Lassiter G, et al. Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques. *Am J Transplant.* 2022;22(1):46-57.
20. Firl DJ, Lassiter G, Hirose T, et al. Clinical and molecular correlation defines activity of physiological pathways in life-sustaining kidney xenotransplantation. *Nat Commun.* 2023;14(1):3022.
21. Anand RP, Layer JV, Heja D, et al. Design and testing of a humanized porcine donor for xenotransplantation. *Nature.* 2023;622(7982):393-401.
22. Swindle MM, Thompson RP, Carabello BA, et al. Heritable ventricular septal defect in Yucatan miniature swine. *Lab Anim Sci.* 1990;40(2):155-161.
23. Ho SY, Thompson RP, Gibbs SR, Swindle MM, Anderson RH. Ventricular septal defects in a family of Yucatan miniature pigs. *Int J Cardiol.* 1991;33(3):419-425.
24. Bittmann I, Mihica D, Plesker R, Denner J. Expression of porcine endogenous retroviruses (PERV) in different organs of a pig. *Virology.* 2012;433(2):329-336.
25. Challies CN. Feral pigs (Sus scrofa) on Auckland Island: status, and effects on vegetation and nesting sea birds. *New Zealand journal of zoology.* 1975;2(4):479-490.
26. Robins JH, Matisoo-Smith E, Ross HA. The origins of the feral pigs on the Auckland Islands. *Journal of the Royal Society of New Zealand.* 2003;33(2):561-569.
27. Garkavenko O, Dieckhoff B, Wynyard S, et al. Absence of transmission of potentially xenotic viruses in a prospective pig to primate islet xenotransplantation study. *J Med Virol.* 2008;80(11):2046-2052.
28. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical Benefit of Islet Xenotransplantation for the Treatment of Type 1 Diabetes. *EBioMedicine.* 2016;12:255-262.
29. Wynyard S. Challenges and practical realities of long-term patient follow-up in three xeno-islet clinical trials: The experience in pig islet xenotransplantation trials in New Zealand and Argentina. *Xenotransplantation.* 2020;27(3):e12605.
30. Wynyard S, Nathu D, Garkavenko O, Denner J, Elliott R. Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. *Xenotransplantation.* 2014;21(4):309-323.
31. Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. *Virus research.* 2017;227:34-40.
32. Fiebig U, Krüger L, Denner J. Determination of the Copy Number of Porcine Endogenous Retroviruses (PERV) in Auckland Island Pigs Repeatedly Used for Clinical Xenotransplantation and Elimination of PERV-C. *Microorganisms.* 2024;12(1).

33. Kurome M, Kessler B, Wuensch A, Nagashima H, Wolf E. Nuclear transfer and transgenesis in the pig. *Methods Mol Biol.* 2015;1222:37-59.
34. Egerer S, Fiebig U, Kessler B, et al. Early weaning completely eliminates porcine cytomegalovirus from a newly established pig donor facility for xenotransplantation. *Xenotransplantation.* 2018;25(4):e12449.
35. Yang B, Cui L, Perez-Enciso M, et al. Genome-wide SNP data unveils the globalization of domesticated pigs. *Genet Sel Evol.* 2017;49(1):71.
36. Powell JE, Visscher PM, Goddard ME. Reconciling the analysis of IBD and IBS in complex trait studies. *Nat Rev Genet.* 2010;11(11):800-805.
37. Stirm M, Fonteyne LM, Shashikadze B, et al. A scalable, clinically severe pig model for Duchenne muscular dystrophy. *Dis Model Mech.* 2021;14(12).
38. Stirm M, Shashikadze B, Blutke A, et al. Systemic deletion of DMD exon 51 rescues clinically severe Duchenne muscular dystrophy in a pig model lacking DMD exon 52. *Proc Natl Acad Sci U S A.* 2023;120(29):e2301250120.
39. Thomas WP, Gaber CE, Jacobs GJ, et al. Recommendations for standards in transthoracic two-dimensional echocardiography in the dog and cat. Echocardiography Committee of the Specialty of Cardiology, American College of Veterinary Internal Medicine. *J Vet Intern Med.* 1993;7(4):247-252.
40. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence of absence of asynergy. *Am J Cardiol.* 1976;37(1):7-11.
41. Albl B, Haesner S, Braun-Reichhart C, et al. Tissue Sampling Guides for Porcine Biomedical Models. *Toxicol Pathol.* 2016;44(3):414-420.
42. Lumsden JH, Mullen K. On establishing reference values. *Can J Comp Med.* 1978;42(3):293-301.
43. Kraft W, Dürr UM. *Klinische Labordiagnostik in der Tiermedizin.* Schattauer Verlag; 2013.
44. Molina DK, DiMaio VJ. Normal Organ Weights in Women: Part I-The Heart. *Am J Forensic Med Pathol.* 2015;36(3):176-181.
45. Goerlich CE, Griffith B, Hanna P, et al. The growth of xenotransplanted hearts can be reduced with growth hormone receptor knockout pig donors. *J Thorac Cardiovasc Surg.* 2023;165(2):e69-e81.
46. Denner J, Längin M, Reichart B, et al. Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival. *Sci Rep.* 2020;10(1):17531.
47. Denner J. Virus Safety of Xenotransplantation. *Viruses.* 2022;14(9):1926.
48. Liu Y, Niu Y, Ma X, et al. Porcine endogenous retrovirus: classification, molecular structure, regulation, function, and potential risk in xenotransplantation. *Funct Integr Genomics.* 2023;23(1):60.
49. Denner J. How active are porcine endogenous retroviruses (PERVs)? *Viruses.* 2016;8(8):215.
50. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med.* 1997;3(3):282-286.
51. Denner J. The origin of porcine endogenous retroviruses (PERVs). *Arch Virol.* 2021;166(4):1007-1013.
52. Güell M, Niu D, Kan Y, et al. PERV inactivation is necessary to guarantee absence of pig-to-patient PERVs transmission in xenotransplantation. *Xenotransplantation.* 2017;24(6):e12366.
53. Harrison I, Takeuchi Y, Bartosch B, Stoye JP. Determinants of high titer in recombinant porcine endogenous retroviruses. *J Virol.* 2004;78(24):13871-13879.
54. Hedrick PW, Garcia-Dorado A. Understanding Inbreeding Depression, Purging, and Genetic Rescue. *Trends Ecol Evol.* 2016;31(12):940-952.

55. Markljung E, Braunschweig MH, Karlskov-Mortensen P, et al. Genome-wide identification of quantitative trait loci in a cross between Hampshire and Landrace II: meat quality traits. *BMC Genet.* 2008;9:22.
56. Ma X, Li P, Zhu M, et al. Genome-wide association analysis reveals genomic regions on Chromosome 13 affecting litter size and candidate genes for uterine horn length in Erhualian pigs. *Animal.* 2018;12(12):2453-2461.
57. Ladowski JM, Hara H, Cooper DKC. The Role of SLAs in Xenotransplantation. *Transplantation.* 2021;105(2):300-307.
58. Peña JR, Saidman SL. Anti-HLA antibody testing in hematology patients. *Am J Hematol.* 2015;90(4):361-364.
59. Ladowski JM, Hara H, Cooper DK. The role of SLAs in xenotransplantation. *Transplantation.* 2021;105(2):300-307.
60. Lei T, Chen L, Wang K, et al. Genetic engineering of pigs for xenotransplantation to overcome immune rejection and physiological incompatibilities: The first clinical steps. *Frontiers in Immunology.* 2022;13:1031185.
61. Fu R, Fang M, Xu K, et al. Generation of GGTA1−/− β2M−/− CIITA−/− pigs using CRISPR/Cas9 technology to alleviate xenogeneic immune reactions. *Transplantation.* 2020;104(8):1566-1573.
62. Sake HJ, Frenzel A, Lucas-Hahn A, et al. Possible detrimental effects of beta-2-microglobulin knockout in pigs. *Xenotransplantation.* 2019;26(6):e12525.
63. Hammer SE, Ho CS, Ando A, et al. Importance of the Major Histocompatibility Complex (Swine Leukocyte Antigen) in Swine Health and Biomedical Research. *Annu Rev Anim Biosci.* 2020;8:171-198.
64. Hammer SE, Duckova T, Groiss S, et al. Comparative analysis of swine leukocyte antigen gene diversity in European farmed pigs. *Anim Genet.* 2021;52(4):523-531.
65. Choi M-K, Le MT, Cho H, et al. Determination of complete sequence information of the human ABO blood group orthologous gene in pigs and breed difference in blood type frequencies. *Gene.* 2018;640:1-5.
66. Smood B, Hara H, Schoel LJ, Cooper DKC. Genetically-engineered pigs as sources for clinical red blood cell transfusion: What pathobiological barriers need to be overcome? *Blood Rev.* 2019;35:7-17.
67. Smith DM, Newhouse M, Naziruddin B, Kresie L. Blood groups and transfusions in pigs. *Xenotransplantation.* 2006;13(3):186-194.

IV. DISCUSSION

Heart failure is a growing epidemic throughout the entire world (BRAUNWALD, 1997). The best treatment option for advanced heart failure is a heart transplant, though this option is often unavailable due to a shortage in donor organ supply. Only 62.1% of people on the waiting list get transplanted according to UNOS data (BAKHTIYAR et al., 2020). Mechanical assist devices have led to less fatalities due to heart failure and are an option as bridging technology. Though many patients never get a heart transplant after being on MCS. In these patients the bridging technology becomes a destination therapy (POTAPOV et al., 2019; FELIX et al., 2020). Further applying MCS before a heart transplant increases the risk of graft failure. Using it as a destination therapy has a far worse outcome than transplantation itself, with bleeding, infection and coagulopathy as the leading complications and a 5-year survival of 45% in BiVADs in a review of 14 papers (FARAG et al., 2021) compared to a 5-year survival of 92.1-74.3% in heart transplantation worldwide, depending on the original heart pathology (HSICH et al., 2022). Attempts to alleviate the situation have been made by a new allocation system in the United States 2018, using hearts after circulatory death and those of hepatitis C patients (AWAD et al., 2022). Further patients are selected for a good outcome before transplantation, using a heart failure survival prognosis score, medical personnel are being educated and bureaucratic hurdles are being broken down. But still all these efforts haven't led to a satisfactory result (AWAD et al., 2022).

Therefore, xenotransplantation is a hope to fill the immense lack of donor organs for advanced heart failure patients. A steady supply of qualitatively good organs would alleviate the situation and make heart transplants of mismatched patients or patients with hepatitis C obsolete. Immense efforts in research have been undertaken, first utilizing nonhuman primates and later pigs (REICHART et al., 2021). To overcome the immune barrier, pigs have been genetically modified with multiple transgenes, knocking out major targets for the hyperacute immune reaction and implementing human surface antigens for complement regulation and coagulation regulation. Also, the differences in anatomy and physiology, as well as the differences in organ size between species has been accommodated for. Microbiological pathogens were identified, screened for and eliminated in DPF herds, leaving pig organs at less risk of infecting the recipient (LANGIN et al.,

2018; REICHART et al., 2023). Identifying the optimal donor breed has proven difficult for multiple reasons. Here we propose Auckland Island pigs as superior to other breeds for the use in pig to human xenotransplantation.

1. Establishment of a breeding colony

Using male and female kidney cell lines kindly provided by NZeno, we used somatic cell nuclear transfer to generate clones, that were then transferred into 3 sows, two of which became pregnant. They gave birth to 3 male and 4 female Auckland island piglets that were raised to adulthood under SPF conditions in the Center for Innovative Medical Models (CiMM). Two boars and three sows were naturally mated and posed as founders for the F1 generation. From this offspring we were able to generate an F2 generation, also by natural mating. They exhibit good fertility and did not show any sign of pathologies even at 5 years of age.

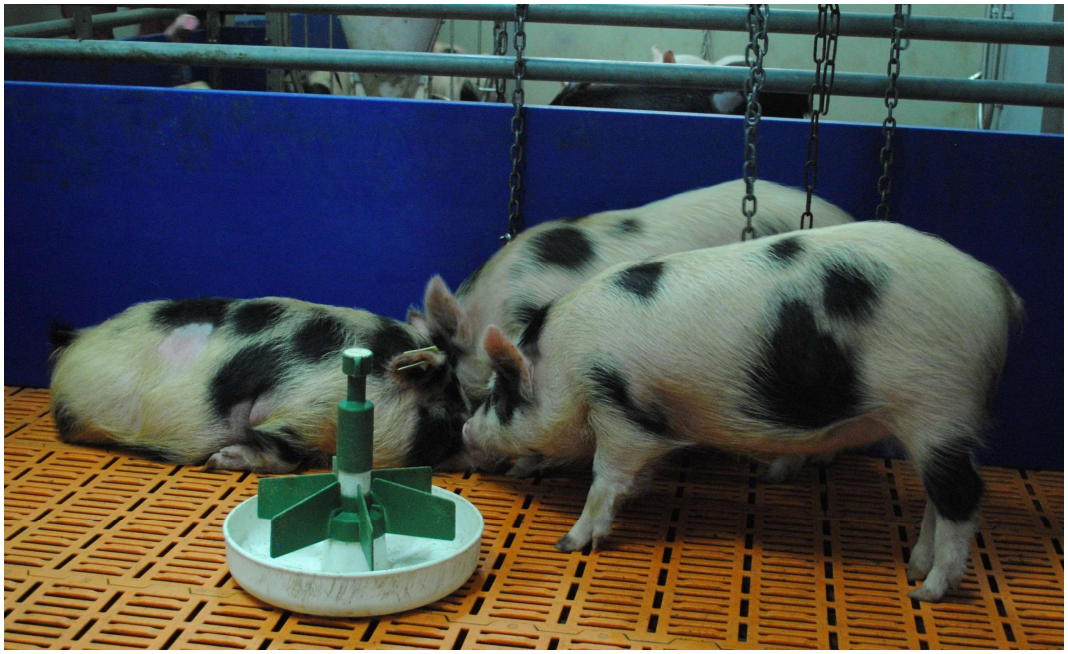

Figure 4: 3 Auckland Island Pigs living in the Center for Innovative Medical Models

2. Study of inbreeding and pathologies

A concern using an island population of pigs may be inbreeding depression. They have been a closed population for over 200 years, with the last confirmed release in 1842. Challies reported at least one major decline in population of the Auckland Island pigs during the 1860s, with them dying out locally on the northwestern part of the island for unknown reasons (CHALLIES, 1975). Multiple genetic methods,

such as analyzing mitochondrial D-Loop DNA, using microsatellites or SNPs have revealed a high degree of inbreeding (GONGORA et al., 2004; FAN et al., 2005). Keeping them in a closed DPF herd will support further inbreeding, as the genetic pool is made up of a closed set of founder animals. Inbreeding has been linked to a multitude of pathologies (JOAQUIM et al., 2019), like heart defects in an Australian herd of "Westran" pigs and reduced fertility (HOLYOAKE et al., 2006).

3. ROH

Genomic analyses revealed low variability and high degree of homozygosity, as could be expected in island population. We performed a SNP Analysis followed by an analysis of the runs of homozygosity (ROH). Here we also found levels of ROH, much greater than modern domestic pig breeds, revealing a high degree of inbreeding (YANG et al., 2017). However, none of our analyses of the phenotype revealed any sign of inbreeding depression. Pigs are in good health and show good fertility, even after 5 years in our facility. This resilience might be attributed to a gradual rise in genetic homozygosity, with new pools of pigs being introduced over time in the first years. Further the elimination of deleterious alleles through natural selection on the island is feasible (HEDRICK & GARCIA-DORADO, 2016).

Notably chromosomes 6 and 13 show lower levels of ROH than the other chromosomes. These chromosomes harbor genes associated with meat quality traits (MARKLJUNG et al., 2008) and characteristics such as litter size and uterine horn length (MA et al., 2018). It can be speculated, that these chromosomes also harbor genes, essential to the survival of the pigs. The coming generations need to be monitored for signs of inbreeding depression.

4. Organ function

Organ function has been extensively studied in 2 founders, 25 animals of the F1 generation and 7 animals of the F2 generation. Serum parameters show slight differences to other pig breeds but indicate no sign of organ malfunction. Echocardiography was performed to further asses the health of the heart in vivo, with only minimal alterations in the pulmonary valve, except for one animal with pericarditis. This contrasts with other minipig lines, like the Yucatan pigs, that have a predisposition for ventricular septal defects and patent foramen ovale (SWINDLE et al., 1990). Also, the Australian "Westran" pigs have a high prevalence of heart

disease (HOLYOAKE et al., 2006). Some modern domestic pig breeds have a high prevalence of sudden cardiac death. This can be attributed to LV stiffening by increase in myocardial collagen, a shift from compliant N2BA titin isoform towards the stiff N2B, and a marked elevation of aortic blood pressure (VAN ESSEN et al., 2018). Whether this plays a role in the longevity of these organs during xenotransplantation has not been studied. Therefore, Auckland Island pigs seem preferable to other minipig lines due to their good cardiac health and presumably higher longevity compared to domestic pig breeds. Additionally, we created reference values for our Auckland Island pigs in 2D echocardiography, the key tool to diagnosing heart pathologies.

5. Organ size

Size matching has already been a challenge in human allotransplantation, with a multitude of approaches. Lastly the International Society for Heart and Lung Transplantation recommends the use of predicted heart mass (PHM) as good predictor for appropriate size matching (KHUSH et al., 2019). In adult humans, a normal heart weighs from 156 to 422 g in women (MOLINA & DIMAIO, 2015) and from 188 to 575 g in men (MOLINA & DIMAIO, 2012). Notably, larger domestic pig breeds, with adult weights reaching up to 350 kg, have heart weights of over 600 g (VAN ESSEN et al., 2018), too big for human transplantation.

In a preclinical study, Längin et al. has transplanted the hearts of juvenile crossbred pigs aged 6-12 weeks into weight matched baboons. The Xenograft kept on growing in the baboon, leading to failure after 28 days (LANGIN et al., 2018). Similar observations have been made in other studies of kidney transplantation. This suggests that even when isolated at early age, porcine organs retain the capacity to grow to their mature size in the recipient (DENNER et al., 2018). Hence size management is key to avoid xenograft overgrowth.

In the aforementioned study, size management was accomplished by using the prodrug tacrolimus, an m-TOR inhibitor, early weening of cortisol and reduction of the blood pressure (LANGIN et al., 2018). This method stands in contrast with a non-nephrotoxic immunosuppression needed for the recipient. Whether this approach is practicable in a clinical setting is questionable.

In the first compassionate pig to human heart transplantation in Maryland, a *GHR*-knockout was used to tackle the problem of xenograft overgrowth (GRIFFITH et al., 2022). However, *GHR*-knockout leads to various pathophysiological

complications (RIEDEL et al., 2020; HINRICHS et al., 2021; SHASHIKADZE et al., 2023) in the donor pig and the long-term effects on their organ health still have to be studied. It can be speculated, that a modern domestic pig breed with already impaired cardiac function at maturity, might be affected negatively by the metabolic changes. These include obesity (HINRICHS et al., 2018), which is a known risk factor for heart disease and heart failure in humans.

Yucatan pigs have already been established as donors in kidney xenotransplantation (MA et al., 2022; FIRL et al., 2023) trials and have median weight of 83 kg resembling human proportions (PANEPINTO et al., 1978). However, they are prone to ventricular septal defects and patent foramen ovale (HO et al., 1991). As they originate from wild boars of the Mexican peninsula of Yucatan, they don't have a naturally high standard of hygiene and inbreeding is likely to occur in a laboratory setting. Additionally, Yucatan pigs are known for a high copy number of porcine endogenous retroviruses in their genome (DENNER & SCHUURMAN, 2021).

Therefore, finding a donor breed with proportions closely resembling humans, without the tendency to functional defects or high prevalence of pathogenic microorganisms is key. In the wild, adult Auckland Island boars were reported to weigh an average of 41.7 kg while adult sows weigh about 37.3 kg (CHALLIES, 1975). On the other hand, provided with a regular diet, adult Auckland Island pigs bred in Munich are similar to those bred by NZeno Limited in New Zealand where they weigh 100–140 kg for adult males and 65–104 kg for adult sows (unpublished communication). Based on our findings, the hearts of adult Auckland Island pigs aged 9–12 months demonstrate an optimal weight and size for potential transplantation into adult humans.

For the matter of organ size, no medical intervention or genetic alteration is needed, as Auckland Island pigs are well suited size match in maturity and no post implant organ growth can be expected. Further Auckland Island pigs are not pruned to heart defects and show good cardiac function and good health for an extended period.

Using 2D transthoracic echocardiography, we can predict the heart mass more accurately, than using weight or height alone. We have also provided reference values for echocardiography. Hence donor pigs can be effectively screened for pathologies using echocardiography prior to transplantation. Also, a matching of heart mass between pig and human is feasible prior to transplantation.

6. Microbiological safety

Cross-species transmission of viruses to the recipient is a major concern in Xenotransplantation. In the first pig to human heart transplantation, porcine cytomegalovirus (PCMV) was detected in the recipient (REARDON, 2022). As this virus is known to significantly promote graft failure in baboons in preclinical studies (DENNER et al., 2020), it might have been a contributing factor to the patient's demise. However, early weaning from the sow and consequent testing of the piglets with PCR, have been shown to create PCMV free pig herds. With modern screening methods it is possible to generate DPF herds, free of most pathogens relevant for xenotransplantation, like the Center for Innovative Medical Models in Munich.

This strategy doesn't apply for porcine endogenous retroviruses, as they are integrated into every pig's genome. Most retroviruses in the genome are ancient and have been inactive for a long time, but PERVs have been detected in altering copy numbers in different tissues, suggesting active infection of cells in the pig (DENNER, 2016). While PERV A and PERV B can cross the species barrier to human cells, PERV-C can exclusively infect porcine cells. So far, only infection of human 293 cells has been shown in vitro, but no infection could be detected in clinical trials (DENNER, 2022). However, recombinants of PERV-A and C have been reported to be 500-fold more infective to human cells.

Using PERV-C free animals is crucial to reduce the possibility of infection and it is mandated by the Food and Drug Administration (FDA) for xenotransplantation. Various strategies have been implemented to reach this goal, like knocking out copies of PERV-C using CRISPR Cas9 (NIU et al., 2017), employing antiretroviral drugs or vaccinating the recipient against PERV. However lesser genetic modifications are preferable for xenotransplantation, as breeding strategies become less complicated, and adverse influences of modifications amongst one another can be ruled out more easily (KEMTER et al., 2020). Also, antiviral drugs might promote cross reactions with other drugs in the immunosuppression regimen. Using a pig breed with a low copy number or no active copies of PERV-C seems preferable.

Auckland island pigs have been kept under DPF conditions for years and screened for various pathogens including PERV. As they have a low copy number of PERV-C, that is lacking a specific sequence, found in infective PERV-C, they have been declared as PERV-C "null" (GARKAVENKO et al., 2008). During clinical trials of pancreatic islet xenotransplantation, no PERVs were transmitted to the human recipient (WYNYARD et al., 2014). In contrast to other minipig breeds with a very high copy number of PERV in their genome, Auckland Island pigs seem to be preferable tissue and organ donors for xenotransplantation.

7. Study of SLA haplotype

As mentioned earlier, crossmatching the SLA and HLA antigens will play an important role in choosing the right donor. SLA and HLA studies by Hundrieser et al. have shown different degrees of reaction towards different SLA haplotypes (HUNDRIESER et al., 2019).

Knocking out SLAs from donor pigs has been considered to avoid immune rejection of the xenograft. However, immunosuppression of the pig through this modification might render it at risk for infectious complications (HAMMER et al., 2020). Without an MHC receptor the cell cannot present viral antigen, so a viral infection of the xenograft might go undetected by the immune system. Also, SLA deficient organs would not be able to induce a tolerance, which makes a NK-mediated cytotoxicity more likely (HAMMER et al., 2020).

Alternatively, site-specific SLA mutations are recommended to avoid the reactivity with anti-HLA antibodies. These have already been shown in vitro to reduce antibody binding. Their effect on the immune reaction in vivo remains to be shown. Moreover, the careful detection of anti-SLA antibodies in the xenograft recipients can assist in reducing cross-reactivity issues (HAMMER et al., 2020). Implementing these strategies requires a precise classification of SLAs in the donor pig breed. SLAs are hyperpolymorphic genes, with over 150 loci encoded in the SLA region at chromosome 7, at least 120 of which are believed to be functional. SLAs are categorized into SLA class I (SLA-1, SLA-2, SLA-3), class II (DRB1, DQB1, DQA), and class III molecules. To date, at least 50 class I and 37 class II SLA haplotypes have been identified (HAMMER et al., 2021), and the database continues to expand. Intriguingly, in our Auckland Island pig colony in Munich, the entire SLA region on chromosome 7 was found to be homozygous in all animals.

Although MHC haplotypes could be determined for numerous SLA genes, it failed for some, such as SLA-3. This limitation might be attributed to preliminary pig genome annotations that are currently under revision for the chromosomal SLA gene complex (Sabine E. Hammer, personal communication 2024). Nevertheless, the homozygosity of SLAs in our Auckland Island pigs simplifies potential strategies to overcome SLA-mediated xenograft rejection in preclinical and clinical trials.

Matching blood types between donor and recipient is an important issue in allotransplantation. So far in xenotransplantation only pigs with blood type O have been used, as they produce no surface antigen, that cross-reacts with the human alloantibodies against A (SMOOD et al., 2019). The problem of matching the blood type, that has so far been a limiting factor, is ruled out completely.

V. FURTHER RESEARCH

So far only a colony of wild-type (WT) Auckland Island pigs has been established to study their traits, physiology, and health. Some obstacles of xenotransplantation, like the organ overgrowth or the infecting the recipient through the xenograft with PERV-C are eliminated by their natural traits. However, they still need some genetic modifications to overcome hyperacute rejection through the major carbohydrate antigens, to modulate the complement cascade and the human coagulation pathways. We have investigated their health over 3 generations in our facility. Even though the ROHs show a homogenous genetic relationship we have found no signs of inbreeding depression. There still is some genetic variability, especially on chromosomes 6 and 13. Further inbreeding is bound to occur, due to the small population size and limited gene pool. Therefore, the next generations need close monitoring for any signs of inbreeding depression. The reaction of the human recipients on the SLA haplotype of our Auckland Island pigs is not yet known. Possible reactions need to be studied and genetic modifications to circumvent reactions by the recipient need to be considered.

VI. OUTLOOK

In genetically modifying the Auckland Island pigs, they could become an excellent genetic background as organ donors for xenotransplantation. Many obstacles of xenotransplantation are met by their natural traits and need no further intervention. Hence genetic modifications needed are fewer than for other pig breeds. Also, the treatment for the recipient will not be as extensive.

In bringing xenotransplantation into clinical practice, the desperate need for donor organs may be met. Additionally, other types of heart disease might be treated, as organs are readily available. Also, patients with a bad prognosis or high cross reactivity to HLA would get a chance for a transplant. The global epidemic of heart disease might demand less victims through this great achievement in science.

VII. SUMMARY

Heart failure is an epidemic claiming thousands of deaths every year. Though new treatment options, like mechanical circulatory support devices, are available, heart transplantation remains the best option. Limiting factor is a shortage in donor organs, resulting in many people dying on the waiting list for a transplant every year.

Xenotransplantation is one hope to alleviate this shortage, by closing the gap with porcine hearts. Pigs were identified as the ideal species, as monkeys are critical through possible viral infections and for ethical reasons. Major advances have been made in genetically modifying pigs to overcome immune barriers. Knockout of the surface antigens Galactose-α(1,3)-galactose (αGal), N-glycolylneuraminic acid (Neu5G) and a glycan corresponding to the human Sd(a) blood group antigen in pigs, made it possible to overcome a hyperacute rejection, triggered by natural antibodies every human has. Complement regulation was achieved by introducing the human regulatory proteins CD46 and CD55 into the pig's genome. Human thrombomodulin expression prevents the porcine tissue from coagulopathy.

One of the prerequisites for successful organ xenotransplantation is a reasonable size match between the porcine organ and the recipient's organ to be replaced. A knockout of the growth hormone receptor can reduce the size of farmed pigs to be more fitting to humans. However, this comes with major alterations in the pig. Therefore, the selection of a naturally smaller genetic background of source pigs is advantageous.

In this study, we investigated body and organ growth, cardiac function, and genetic diversity of a colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich. Male and female Auckland Island pig kidney cells (selected to be free of porcine endogenous retrovirus C) were imported from New Zealand, and founder animals were established by somatic cell nuclear transfer (SCNT). Morphologically, Auckland Island pigs have smaller body stature compared to many domestic pig breeds, rendering their organ dimensions well-suited for human transplantation. Furthermore, echocardiography assessments of Auckland Island pig hearts indicated normal structure and functioning across various age groups throughout the study. Single nucleotide polymorphism (SNP) analysis revealed higher runs of homozygosity (ROH) in Auckland Island pigs

compared to other domestic pig breeds and demonstrated that the entire locus coding the swine leukocyte antigens (SLAs) was homozygous. Based on these findings, Auckland Island pigs represent a promising genetic background for organ xenotransplantation.

VIII. ZUSAMMENFASSUNG

Genetische Vielfalt, Wachstum und Herzfunktion von Auckland Island Schweinen, einer potenziellen Organ-Quelle für die Xenotransplantation

Herzinsuffizienz ist eine Epidemie, die jedes Jahr Tausende von Todesfällen fordert. Obwohl neue Behandlungsmöglichkeiten wie maschinelle Kreislaufunterstützungssysteme zur Verfügung stehen, bleibt die Herztransplantation die beste Option. Ein limitierender Faktor ist der Mangel an Spenderorganen, der dazu führt, dass jedes Jahr viele Menschen auf der Warteliste für eine Transplantation sterben.

Die Xenotransplantation ist eine Hoffnung, diesen Mangel zu beheben, indem die Lücke mit Schweineherzen geschlossen wird. Schweine wurden als die ideale Spezies identifiziert, da Affen aufgrund möglicher Virusinfektionen und aus ethischen Gründen kritisch sind. Bei der gentechnischen Veränderung von Schweinen zur Überwindung von Immunbarrieren wurden bereits große Fortschritte erzielt. Durch Knockout der Oberflächenantigene Galaktose-α(1,3)-Galaktose (αGal), N-Glykolylneuraminsäure (Neu5G) und eines Glykans, das dem menschlichen Sd(a)-Blutgruppenantigen entspricht, konnte eine hyperakute Abstoßung, die durch natürliche Antikörper, die jeder Mensch besitzt, ausgelöst wird, bei Schweinen überwunden werden. Die Komplementregulierung wurde durch die Einführung der menschlichen Regulierungsproteine CD46 und CD55 in das Genom des Schweins erreicht. Die Expression von menschlichem Thrombomodulin verhindert eine Koagulopathie im Schweinegewebe.

Eine der Voraussetzungen für eine erfolgreiche Xenotransplantation von Organen ist eine angemessene Größenübereinstimmung zwischen dem Schweineorgan und dem zu ersetzenden Organ des Empfängers. Durch Knockout des Wachstumshormonrezeptors kann die Größe von Zuchtschweinen so reduziert werden, dass sie besser zum Menschen passt. Dies ist jedoch mit erheblichen Veränderungen beim Schwein verbunden. Daher ist die Auswahl eines natürlich kleineren genetischen Hintergrunds des Donorschweines von Vorteil.

In dieser Studie untersuchten wir das Körper- und Organwachstum, die Herzfunktion und die genetische Vielfalt einer Kolonie von Auckland Island Schweinen, die am Center for Innovative Medical Models (CiMM) der LMU

München etabliert wurde. Männliche und weibliche Nierenzellen des Auckland Island Schweins (ausgewählt, um frei vom porzinen endogenen Retrovirus C zu sein) wurden aus Neuseeland importiert und Gründertiere wurden durch somatischen Zellkerntransfer (SCNT) etabliert. Morphologisch gesehen haben Auckland-Island-Schweine im Vergleich zu vielen Hausschweinrassen einen kleineren Körperbau, so dass sich ihre Organdimensionen gut für die Transplantation beim Menschen eignen. Darüber hinaus zeigten die echokardiographischen Untersuchungen der Herzen der Auckland-Island Schweine während der gesamten Studie eine normale Struktur und Funktion in verschiedenen Altersgruppen. Die Analyse von Einzelnukleotid-Polymorphismen (SNP) ergab bei den Auckland-Island Schweinen im Vergleich zu anderen Hausschweinrassen eine höhere Homozygotie (ROH) und zeigte, dass der gesamte Locus, der die Schweineleukozyten-Antigene (SLAs) kodiert, homozygot war. Auf der Grundlage dieser Ergebnisse stellen Auckland Island Schweine einen vielversprechenden genetischen Hintergrund für die Xenotransplantation von Organen dar.

IX. REFERENCES

Administration HRS. https://www.organdonor.gov/learn/organ-donation-statistics/detailed-description#fig1. 2024: 12.05.

Ali A, Kemter E, Wolf E. Advances in Organ and Tissue Xenotransplantation. Annu Rev Anim Biosci 2024; 12: 369-90.

Allan JS. The risk of using baboons as transplant donors. Exogenous and endogenous viruses. Ann N Y Acad Sci 1998; 862: 87-99.

Arabia FA, Cantor RS, Koehl DA, Kasirajan V, Gregoric I, Moriguchi JD, Esmailian F, Ramzy D, Chung JS, Czer LS, Kobashigawa JA, Smith RG, Kirklin JK. Interagency registry for mechanically assisted circulatory support report on the total artificial heart. J Heart Lung Transplant 2018; 37: 1304-12.

Atti V, Narayanan MA, Patel B, Balla S, Siddique A, Lundgren S, Velagapudi P. A Comprehensive Review of Mechanical Circulatory Support Devices. Heart Int 2022; 16: 37-48.

Authors/Task Force M, McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Bohm M, Burri H, Butler J, Celutkiene J, Chioncel O, Cleland JGF, Coats AJS, Crespo-Leiro MG, Farmakis D, Gilard M, Heymans S, Hoes AW, Jaarsma T, Jankowska EA, Lainscak M, Lam CSP, Lyon AR, McMurray JJV, Mebazaa A, Mindham R, Muneretto C, Francesco Piepoli M, Price S, Rosano GMC, Ruschitzka F, Kathrine Skibelund A, Group ESCSD. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). With the special contribution of the Heart Failure Association (HFA) of the ESC. Eur J Heart Fail 2022; 24: 4-131.

Awad MA, Shah A, Griffith BP. Current status and outcomes in heart transplantation: a narrative review. Rev Cardiovasc Med 2022; 23: 11.

Bailey LL, Nehlsen-Cannarella SL, Concepcion W, Jolley WB. Baboon-to-human cardiac xenotransplantation in a neonate. JAMA 1985; 254: 3321-9.

Bakhtiyar SS, Godfrey EL, Ahmed S, Lamba H, Morgan J, Loor G, Civitello A, Cheema FH, Etheridge WB, Goss J, Rana A. Survival on the Heart Transplant Waiting List. JAMA Cardiol 2020; 5: 1227-35.

Bittmann I, Mihica D, Plesker R, Denner J. Expression of porcine endogenous retroviruses (PERV) in different organs of a pig. Virology 2012; 433: 329-36.

Bosse M, Megens HJ, Madsen O, Paudel Y, Frantz LA, Schook LB, Crooijmans RP, Groenen MA. Regions of homozygosity in the porcine genome: consequence of demography and the recombination landscape. PLoS Genet 2012; 8: e1003100.

Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med 1997; 337: 1360-9.

Calne RY, Rolles K, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Henderson RG, Aziz S, Lewis P. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. Lancet 1979; 2: 1033-6.

Cengiz N, Wareham CS. Ethical considerations in xenotransplantation: a review. Curr Opin Organ Transplant 2020; 25: 483-8.

Challies CN. Feral pigs (Sus scrofa) on Auckland Island: Status, and effects on vegetation and nesting sea birds. New Zealand Journal of Zoology 1975; 2: 479-90.

Chen KY, Liu J, Ren EC. Structural and functional distinctiveness of HLA-A2 allelic variants. Immunol Res 2012; 53: 182-90.

Choi MK, Le MT, Cho H, Yum J, Kang M, Song H, Kim JH, Chung HJ, Hong K, Park C. Determination of complete sequence information of the human ABO blood group orthologous gene in pigs and breed difference in blood type frequencies. Gene 2018; 640: 1-5.

Cook JA, Shah KB, Quader MA, Cooke RH, Kasirajan V, Rao KK, Smallfield MC, Tchoukina I, Tang DG. The total artificial heart. J Thorac Dis 2015; 7: 2172-80.

Cooper DK, Good AH, Koren E, Oriol R, Malcolm AJ, Ippolito RM, Neethling FA, Ye Y, Romano E, Zuhdi N. Identification of alpha-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transpl Immunol 1993; 1: 198-205.

Cooper DK, Dorling A, Pierson RN, 3rd, Rees M, Seebach J, Yazer M, Ohdan H, Awwad M, Ayares D. Alpha1,3-galactosyltransferase gene-knockout pigs for xenotransplantation: where do we go from here? Transplantation 2007; 84: 1-7.

Cooper DKC, Foote JB, Javed M, Nguyen HQ, Bikhet MH, Hansen-Estruch C, Ayares D, Hara H. Initial evidence that blockade of the CD40/CD154 costimulation pathway alone is sufficient as maintenance therapy in xenotransplantation. Xenotransplantation 2021; 28: e12721.

Crespo-Leiro MG, Metra M, Lund LH, Milicic D, Costanzo MR, Filippatos G, Gustafsson F, Tsui S, Barge-Caballero E, De Jonge N, Frigerio M, Hamdan R, Hasin T, Hulsmann M, Nalbantgil S, Potena L, Bauersachs J, Gkouziouta A, Ruhparwar A, Ristic AD, Straburzynska-Migaj E, McDonagh T, Seferovic P, Ruschitzka F. Advanced heart failure: a position statement of the Heart Failure Association of the European Society of Cardiology. Eur J Heart Fail 2018; 20: 1505-35.

Crick SJ, Sheppard MN, Ho SY, Gebstein L, Anderson RH. Anatomy of the pig heart: comparisons with normal human cardiac structure. J Anat 1998; 193 (Pt 1): 105-19.

de By T, Schoenrath F, Veen KM, Mohacsi P, Stein J, Alkhamees KMM, Anastasiadis K, Berhnardt A, Beyersdorf F, Caliskan K, Reineke D, Damman K, Fiane A, Gkouziouta A, Gollmann-Tepekoylu C, Gustafsson F, Hulman M, Iacovoni A, Loforte A, Merkely B, Musumeci F, Nemec P, Netuka I, Ozbaran M, Potapov E, Pya Y, Rabago G, Ramjankhan F, Reichenspurner H, Saeed D, Sandoval E, Stockman B, Vanderheyden M, Tops L, Wahlers T, Zembala M, Zimpfer D, Carrel T, Gummert J, Meyns B. The European Registry for Patients with Mechanical Circulatory Support of the European Association for Cardio-Thoracic Surgery: third report. Eur J Cardiothorac Surg 2022; 62.

Denner J. How Active Are Porcine Endogenous Retroviruses (PERVs)? Viruses 2016; 8.

Denner J, Reichart B, Längin M. Does size matter? Xenotransplantation 2018; 25: e12383.

Denner J, Langin M, Reichart B, Kruger L, Fiebig U, Mokelke M, Radan J, Mayr T, Milusev A, Luther F, Sorvillo N, Rieben R, Brenner P, Walz C, Wolf E, Roshani B, Stahl-Hennig C, Abicht JM. Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival. Sci Rep 2020; 10: 17531.

Denner J. Sensitive detection systems for infectious agents in xenotransplantation. Xenotransplantation 2020a: e12594.

Denner J. By definition. Xenotransplantation 2020b; 27: e12599.

Denner J. Porcine Lymphotropic Herpesviruses (PLHVs) and Xenotranplantation. Viruses 2021; 13.

Denner J, Schuurman HJ. High Prevalence of Recombinant Porcine Endogenous Retroviruses (PERV-A/Cs) in Minipigs: A Review on Origin and Presence. Viruses 2021; 13.

Denner J. Virus Safety of Xenotransplantation. Viruses 2022; 14.

Diamond LE, Quinn CM, Martin MJ, Lawson J, Platt JL, Logan JS. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. Transplantation 2001; 71: 132-42.

Edwards JL, Schrick FN, McCracken MD, van Amstel SR, Hopkins FM, Welborn MG, Davies CJ. Cloning adult farm animals: a review of the possibilities and problems associated with somatic cell nuclear transfer. Am J Reprod Immunol 2003; 50: 113-23.

Egerer S, Fiebig U, Kessler B, Zakhartchenko V, Kurome M, Reichart B, Kupatt C, Klymiuk N, Wolf E, Denner J, Bahr A. Early weaning completely eliminates porcine cytomegalovirus from a newly established pig donor facility for xenotransplantation. Xenotransplantation 2018; 25: e12449.

Emmons-Bell S, Johnson C, Roth G. Prevalence, incidence and survival of heart failure: a systematic review. Heart 2022; 108: 1351-60.

Etheredge HR. Assessing Global Organ Donation Policies: Opt-In vs Opt-Out. Risk Manag Healthc Policy 2021; 14: 1985-98.

Fan B, Gongora J, Chen Y, Garkavenko O, Li K, Moran C. Population genetic variability and origin of Auckland Island feral pigs. Journal of the Royal Society of New Zealand 2005; 35: 279-85.

Farag J, Woldendorp K, McNamara N, Bannon PG, Marasco SF, Loforte A, Potapov EV. Contemporary outcomes of continuous-flow biventricular assist devices. Ann Cardiothorac Surg 2021; 10: 311-28.

Felix SEA, de Jonge N, Caliskan K, Birim O, Damman K, Kuijpers M, Tops LF, Palmen M, Ramjankhan FZ. The role of long-term mechanical circulatory support in patients with advanced heart failure. Neth Heart J 2020; 28: 115-21.

Firl DJ, Lassiter G, Hirose T, Policastro R, D'Attilio A, Markmann JF, Kawai T, Hall KC. Clinical and molecular correlation defines activity of physiological pathways in life-sustaining kidney xenotransplantation. Nat Commun 2023; 14: 3022.

Ganapathi AM, Lampert BC, Mokadam NA, Emani S, Hasan AK, Tamer R, Whitson BA. Allocation changes in heart transplantation: What has really changed? J Thorac Cardiovasc Surg 2023; 165: 724-33 e7.

Garkavenko O, Wynyard S, Nathu D, Simond D, Muzina M, Muzina Z, Scobie L, Hector RD, Croxson MC, Tan P, Elliott BR. Porcine endogenous retrovirus (PERV) and its transmission characteristics: a study of the New Zealand designated pathogen-free herd. Cell Transplant 2008; 17: 1381-8.

Gerber Y, Weston SA, Redfield MM, Chamberlain AM, Manemann SM, Jiang R, Killian JM, Roger VL. A contemporary appraisal of the heart failure epidemic in Olmsted County, Minnesota, 2000 to 2010. JAMA Intern Med 2015; 175: 996-1004.

GmbH A. https://www.cardiovascular.abbott/de/de/hcp/products/heart-failure/left-ventricular-assist-devices/heartmate-3/about.html. 2024a: 17.05.

GmbH R. https://www.researchgate.net/figure/Auckland-Islands-archipelago_fig1_365783198. 2024b: 17.05.

Gongora J, Fleming P, Spencer PB, Mason R, Garkavenko O, Meyer JN, Droegemueller C, Lee JH, Moran C. Phylogenetic relationships of Australian and New Zealand feral pigs assessed by mitochondrial control region sequence and nuclear GPIP genotype. Mol Phylogenet Evol 2004; 33: 339-48.

Griffith BP, Goerlich CE, Singh AK, Rothblatt M, Lau CL, Shah A, Lorber M, Grazioli A, Saharia KK, Hong SN, Joseph SM, Ayares D, Mohiuddin MM. Genetically Modified Porcine-to-Human Cardiac Xenotransplantation. N Engl J Med 2022; 387: 35-44.

Groenewegen A, Rutten FH, Mosterd A, Hoes AW. Epidemiology of heart failure. Eur J Heart Fail 2020; 22: 1342-56.

Hammer SE, Ho CS, Ando A, Rogel-Gaillard C, Charles M, Tector M, Tector AJ, Lunney JK. Importance of the Major Histocompatibility Complex (Swine Leukocyte Antigen) in Swine Health and Biomedical Research. Annu Rev Anim Biosci 2020; 8: 171-98.

Hammer SE, Duckova T, Groiss S, Stadler M, Jensen-Waern M, Golde WT, Gimsa U, Saalmueller A. Comparative analysis of swine leukocyte antigen gene diversity in European farmed pigs. Anim Genet 2021; 52: 523-31.

Hardy JD, Kurrus FD, Chavez CM, Neely WA, Eraslan S, Turner MD, Fabian LW, Labecki TD. Heart Transplantation in Man. Developmental Studies and Report of a Case. JAMA 1964; 188: 1132-40.

Hawthorne WJ. World first pig-to-human cardiac xenotransplantation. Xenotransplantation 2022; 29: e12733.

Hedrick PW, Garcia-Dorado A. Understanding Inbreeding Depression, Purging, and Genetic Rescue. Trends Ecol Evol 2016; 31: 940-52.

Heidenreich PA, Albert NM, Allen LA, Bluemke DA, Butler J, Fonarow GC, Ikonomidis JS, Khavjou O, Konstam MA, Maddox TM, Nichol G, Pham M, Pina IL, Trogdon JG, American Heart Association Advocacy Coordinating C, Council on Arteriosclerosis T, Vascular B, Council on Cardiovascular R, Intervention, Council on Clinical C, Council on E, Prevention, Stroke C. Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association. Circ Heart Fail 2013; 6: 606-19.

Hinrichs A, Kessler B, Kurome M, Blutke A, Kemter E, Bernau M, Scholz AM, Rathkolb B, Renner S, Bultmann S, Leonhardt H, de Angelis MH, Nagashima H, Hoeflich A, Blum WF, Bidlingmaier M, Wanke R, Dahlhoff M, Wolf E. Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron

syndrome and reveal altered activation of signaling cascades in the liver. Mol Metab 2018; 11: 113-28.

Hinrichs A, Renner S, Bidlingmaier M, Kopchick JJ, Wolf E. MECHANISMS IN ENDOCRINOLOGY: Transient juvenile hypoglycemia in growth hormone receptor deficiency - mechanistic insights from Laron syndrome and tailored animal models. Eur J Endocrinol 2021; 185: R35-R47.

Ho SY, Thompson RP, Gibbs SR, Swindle MM, Anderson RH. Ventricular septal defects in a family of Yucatan miniature pigs. Int J Cardiol 1991; 33: 419-25.

Holyoake PK, Stevenson J, Moran C, Stokes R, Kirk EP, Sugo E, Hawthorne WJ. The occurrence of congenital heart defects in an inbred herd of pigs in Australia. Aust Vet J 2006; 84: 129-33.

Hsich E, Singh TP, Cherikh WS, Harhay MO, Hayes D, Jr., Perch M, Potena L, Sadavarte A, Lindblad K, Zuckermann A, Stehlik J, International Society for H, Lung T. The International thoracic organ transplant registry of the international society for heart and lung transplantation: Thirty-ninth adult heart transplantation report-2022; focus on transplant for restrictive heart disease. J Heart Lung Transplant 2022; 41: 1366-75.

Huang M, Yang B, Chen H, Zhang H, Wu Z, Ai H, Ren J, Huang L. The fine-scale genetic structure and selection signals of Chinese indigenous pigs. Evol Appl 2020; 13: 458-75.

Huckaby LV, Seese LM, Handzel R, Wang Y, Hickey G, Kilic A. Center-level Utilization of Hepatitis C Virus-positive Donors for Orthotopic Heart Transplantation. Transplantation 2021; 105: 2639-45.

Hundrieser J, Hein R, Pokoyski C, Brinkmann A, Duvel H, Dinkel A, Trautewig B, Siegert JF, Romermann D, Petersen B, Schwinzer R. Role of human and porcine MHC DRB1 alleles in determining the intensity of individual human anti-pig T-cell responses. Xenotransplantation 2019; 26: e12523.

Joaquim LB, Chud TCS, Marchesi JAP, Savegnago RP, Buzanskas ME, Zanella R, Cantao ME, Peixoto JO, Ledur MC, Irgang R, Munari DP. Genomic structure of a crossbred Landrace pig population. PLoS One 2019; 14: e0212266.

Keefer CL. Artificial cloning of domestic animals. Proc Natl Acad Sci U S A 2015; 112: 8874-8.

Kemter E, Schnieke A, Fischer K, Cowan PJ, Wolf E. Xeno-organ donor pigs with multiple genetic modifications - the more the better? Curr Opin Genet Dev 2020; 64: 60-5.

Khush KK, Cherikh WS, Chambers DC, Harhay MO, Hayes D, Jr., Hsich E, Meiser B, Potena L, Robinson A, Rossano JW, Sadavarte A, Singh TP, Zuckermann A, Stehlik J, International Society for H, Lung T. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. J Heart Lung Transplant 2019; 38: 1056-66.

Kim JH, Cowger JA, Shah P. The Evolution of Mechanical Circulatory Support. Cardiol Clin 2018; 36: 443-9.

Kurihara C, Kawabori M, Sugiura T, Critsinelis AC, Wang S, Cohn WE, Civitello AB, Frazier OH, Morgan JA. Bridging to a Long-Term Ventricular Assist Device With Short-Term Mechanical Circulatory Support. Artif Organs 2018; 42: 589-96.

Ladowski JM, Hara H, Cooper DKC. The Role of SLAs in Xenotransplantation. Transplantation 2021; 105: 300-7.

Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, Flachskampf FA, Foster E, Goldstein SA, Kuznetsova T, Lancellotti P, Muraru D, Picard MH, Rietzschel ER, Rudski L, Spencer KT, Tsang W, Voigt JU. Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the

European Association of Cardiovascular Imaging. Eur Heart J Cardiovasc Imaging 2015; 16: 233-70.

Langin M, Mayr T, Reichart B, Michel S, Buchholz S, Guethoff S, Dashkevich A, Baehr A, Egerer S, Bauer A, Mihalj M, Panelli A, Issl L, Ying J, Fresch AK, Buttgereit I, Mokelke M, Radan J, Werner F, Lutzmann I, Steen S, Sjoberg T, Paskevicius A, Qiuming L, Sfriso R, Rieben R, Dahlhoff M, Kessler B, Kemter E, Kurome M, Zakhartchenko V, Klett K, Hinkel R, Kupatt C, Falkenau A, Reu S, Ellgass R, Herzog R, Binder U, Wich G, Skerra A, Ayares D, Kind A, Schonmann U, Kaup FJ, Hagl C, Wolf E, Klymiuk N, Brenner P, Abicht JM. Consistent success in life-supporting porcine cardiac xenotransplantation. Nature 2018; 564: 430-3.

Lelovas PP, Kostomitsopoulos NG, Xanthos TT. A comparative anatomic and physiologic overview of the porcine heart. J Am Assoc Lab Anim Sci 2014; 53: 432-8.

Lewis A, Koukoura A, Tsianos GI, Gargavanis AA, Nielsen AA, Vassiliadis E. Organ donation in the US and Europe: The supply vs demand imbalance. Transplant Rev (Orlando) 2021; 35: 100585.

Lund LH, Edwards LB, Kucheryavaya AY, Benden C, Dipchand AI, Goldfarb S, Levvey BJ, Meiser B, Rossano JW, Yusen RD, Stehlik J. The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Heart Transplantation Report--2015; Focus Theme: Early Graft Failure. J Heart Lung Transplant 2015; 34: 1244-54.

Ma D, Hirose T, Lassiter G, Sasaki H, Rosales I, Coe TM, Rickert CG, Matheson R, Colvin RB, Qin W, Kan Y, Layer JV, Paragas VB, Stiede K, Hall KC, Youd ME, Queiroz LM, Westlin WF, Curtis M, Yang L, Markmann JF, Kawai T. Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques. Am J Transplant 2022; 22: 46-57.

Ma X, Li PH, Zhu MX, He LC, Sui SP, Gao S, Su GS, Ding NS, Huang Y, Lu ZQ, Huang XG, Huang RH. Genome-wide association analysis reveals genomic regions on Chromosome 13 affecting litter size and candidate genes for uterine horn length in Erhualian pigs. Animal 2018; 12: 2453-61.

Markljung E, Braunschweig MH, Karlskov-Mortensen P, Bruun CS, Sawera M, Cho IC, Hedebro-Velander I, Josell A, Lundstrom K, von Seth G, Jorgensen CB, Fredholm M, Andersson L. Genome-wide identification of quantitative trait loci in a cross between Hampshire and Landrace II: meat quality traits. BMC Genet 2008; 9: 22.

McCartney SL, Patel C, Del Rio JM. Long-term outcomes and management of the heart transplant recipient. Best Pract Res Clin Anaesthesiol 2017; 31: 237-48.

Mehra MR, Canter CE, Hannan MM, Semigran MJ, Uber PA, Baran DA, Danziger-Isakov L, Kirklin JK, Kirk R, Kushwaha SS, Lund LH, Potena L, Ross HJ, Taylor DO, Verschuuren EAM, Zuckermann A, International Society for Heart Lung Transplantation Infectious Diseases P, Heart F, Transplantation C. The 2016 International Society for Heart Lung Transplantation listing criteria for heart transplantation: A 10-year update. J Heart Lung Transplant 2016; 35: 1-23.

Meyer DM, Rogers JG, Edwards LB, Callahan ER, Webber SA, Johnson MR, Vega JD, Zucker MJ, Cleveland JC, Jr. The future direction of the adult heart allocation system in the United States. Am J Transplant 2015; 15: 44-54.

Mohiuddin MM, Singh AK, Corcoran PC, Thomas Iii ML, Clark T, Lewis BG, Hoyt RF, Eckhaus M, Pierson Iii RN, Belli AJ, Wolf E, Klymiuk N, Phelps C, Reimann KA, Ayares D, Horvath KA. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. Nat Commun 2016; 7: 11138.

Mohiuddin MM, Singh AK, Scobie L, Goerlich CE, Grazioli A, Saharia K, Crossan C, Burke A, Drachenberg C, Oguz C, Zhang T, Lewis B, Hershfeld A, Sentz F, Tatarov I, Mudd S, Braileanu G, Rice K, Paolini JF, Bondensgaard K,

Vaught T, Kuravi K, Sorrells L, Dandro A, Ayares D, Lau C, Griffith BP. Graft dysfunction in compassionate use of genetically engineered pig-to-human cardiac xenotransplantation: a case report. Lancet 2023; 402: 397-410.

Molina DK, DiMaio VJ. Normal organ weights in men: part I-the heart. Am J Forensic Med Pathol 2012; 33: 362-7.

Molina DK, DiMaio VJ. Normal Organ Weights in Women: Part I-The Heart. Am J Forensic Med Pathol 2015; 36: 176-81.

Nellore A, Fishman JA. Donor-derived infections and infectious risk in xenotransplantation and allotransplantation. Xenotransplantation 2018; 25: e12423.

Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, Lesha E, Wang G, Luo Y, Qing Y, Jiao D, Zhao H, Zhou X, Wang S, Wei H, Guell M, Church GM, Yang L. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 2017; 357: 1303-7.

Nunoda S. Impact of pretransplant antibodies on outcomes after heart transplantation. Curr Opin Organ Transplant 2019; 24: 220-6.

Nwakanma LU, Williams JA, Weiss ES, Russell SD, Baumgartner WA, Conte JV. Influence of pretransplant panel-reactive antibody on outcomes in 8,160 heart transplant recipients in recent era. Ann Thorac Surg 2007; 84: 1556-62; discussion 62-3.

Panepinto LM, Phillips RW, Wheeler LR, Will DH. The Yucatan minature pig as a laboratory animal. Lab Anim Sci 1978; 28: 308-13.

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE,

Dai Y, Ayares DL. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 2003; 299: 411-4.

Pierozan CR, Callegari MA, Dias CP, de Souza KL, Gasa J, da Silva CA. Herd-level factors associated with piglet weight at weaning, kilograms of piglets weaned per sow per year and sow feed conversion. Animal 2020; 14: 1283-92.

Pinnelas R, Kobashigawa JA. Ex vivo normothermic perfusion in heart transplantation: a review of the TransMedics((R)) Organ Care System. Future Cardiol 2022; 18: 5-15.

Potapov EV, Antonides C, Crespo-Leiro MG, Combes A, Farber G, Hannan MM, Kukucka M, de Jonge N, Loforte A, Lund LH, Mohacsi P, Morshuis M, Netuka I, Ozbaran M, Pappalardo F, Scandroglio AM, Schweiger M, Tsui S, Zimpfer D, Gustafsson F. 2019 EACTS Expert Consensus on long-term mechanical circulatory support. Eur J Cardiothorac Surg 2019; 56: 230-70.

Prescott MJ. Ethics of primate use. Adv Sci Res 2010; 5: 11-22.

Quader MA, Wolfe LG, Kasirajan V. Heart transplantation outcomes from cardiac arrest-resuscitated donors. J Heart Lung Transplant 2013; 32: 1090-5.

Reardon S. First pig-to-human heart transplant: what can scientists learn? Nature 2022; 601: 305-6.

Reichart B, Langin M, Radan J, Mokelke M, Buttgereit I, Ying J, Fresch AK, Mayr T, Issl L, Buchholz S, Michel S, Ellgass R, Mihalj M, Egerer S, Baehr A, Kessler B, Kemter E, Kurome M, Zakhartchenko V, Steen S, Sjoberg T, Paskevicius A, Kruger L, Fiebig U, Denner J, Godehardt AW, Tonjes RR, Milusev A, Rieben R, Sfriso R, Walz C, Kirchner T, Ayares D, Lampe K, Schonmann U, Hagl C, Wolf E, Klymiuk N, Abicht JM, Brenner P. Pig-to-non-human primate heart transplantation: The final step toward clinical xenotransplantation? J Heart Lung Transplant 2020; 39: 751-7.

Reichart B, Langin M, Denner J, Schwinzer R, Cowan PJ, Wolf E. Pathways to Clinical Cardiac Xenotransplantation. Transplantation 2021; 105: 1930-43.

Reichart B, Cooper DKC, Langin M, Tonjes RR, Pierson RN, Wolf E. Cardiac xenotransplantation: from concept to clinic. Cardiovasc Res 2023; 118: 3499-516.

Riedel EO, Hinrichs A, Kemter E, Dahlhoff M, Backman M, Rathkolb B, Prehn C, Adamski J, Renner S, Blutke A, de Angelis MH, Bidlingmaier M, Schopohl J, Arnold GJ, Frohlich T, Wolf E. Functional changes of the liver in the absence of growth hormone (GH) action - Proteomic and metabolomic insights from a GH receptor deficient pig model. Mol Metab 2020; 36: 100978.

Robins JH, Matisoo-Smith E, Ross HA. The origins of the feral pigs on the Auckland Islands. Journal of the Royal Society of New Zealand 2003; 33: 561-9.

Salter BS, Gross CR, Weiner MM, Dukkipati SR, Serrao GW, Moss N, Anyanwu AC, Burkhoff D, Lala A. Temporary mechanical circulatory support devices: practical considerations for all stakeholders. Nat Rev Cardiol 2023; 20: 263-77.

Shahim B, Kapelios CJ, Savarese G, Lund LH. Global Public Health Burden of Heart Failure: An Updated Review. Card Fail Rev 2023; 9: e11.

Shashikadze B, Franzmeier S, Hofmann I, Kraetzl M, Flenkenthaler F, Blutke A, Frohlich T, Wolf E, Hinrichs A. Structural and proteomic repercussions of growth hormone receptor deficiency on the pituitary gland: Lessons from a translational pig model. J Neuroendocrinol 2023: e13277.

Singh AK, Goerlich CE, Shah AM, Zhang T, Tatarov I, Ayares D, Horvath KA, Mohiuddin MM. Cardiac Xenotransplantation: Progress in Preclinical Models and Prospects for Clinical Translation. Transpl Int 2022; 35: 10171.

Singireddy S, Tully A, Galindo J, Ayares D, Singh AK, Mohiuddin MM. Genetic Engineering of Donor Pig for the First Human Cardiac Xenotransplantation:

Combatting Rejection, Coagulopathy, Inflammation, and Excessive Growth. Curr Cardiol Rep 2023; 25: 1649-56.

Smith DM, Newhouse M, Naziruddin B, Kresie L. Blood groups and transfusions in pigs. Xenotransplantation 2006; 13: 186-94.

Smood B, Hara H, Schoel LJ, Cooper DKC. Genetically-engineered pigs as sources for clinical red blood cell transfusion: What pathobiological barriers need to be overcome? Blood Rev 2019; 35: 7-17.

Society NZRB. https://www.rarebreeds.co.nz/auckislandpigs-bs.html. 2008: 14.05.

Steen S, Paskevicius A, Liao Q, Sjoberg T. Safe orthotopic transplantation of hearts harvested 24 hours after brain death and preserved for 24 hours. Scand Cardiovasc J 2016; 50: 193-200.

Swindle MM, Thompson RP, Carabello BA, Smith AC, Hepburn BJ, Bodison DR, Corin W, Fazel A, Biederman WW, Spinale FG, et al. Heritable ventricular septal defect in Yucatan miniature swine. Lab Anim Sci 1990; 40: 155-61.

Taniguchi S, Cooper DK. Clinical xenotransplantation: past, present and future. Ann R Coll Surg Engl 1997; 79: 13-9.

TRANSPLANTATION GOODA. https://www.transplant-observatory.org/summary/. 2023: 06.24.

Truby LK, Rogers JG. Advanced Heart Failure: Epidemiology, Diagnosis, and Therapeutic Approaches. JACC Heart Fail 2020; 8: 523-36.

University of Maryland SoM. https://www.medschool.umaryland.edu/news/2023/um-medicine-faculty-

scientists-and-clinicians-perform-second-historic-transplant-of-pig-heart-into-patient-with-end-stage-cardiovascular-disease.html. 2023: 13.05.

van Essen GJ, Te Lintel Hekkert M, Sorop O, Heinonen I, van der Velden J, Merkus D, Duncker DJ. Cardiovascular Function of Modern Pigs Does not Comply with Allometric Scaling Laws. Sci Rep 2018; 8: 792.

Vishram-Nielsen JKK, Tomasoni D, Gustafsson F, Metra M. Contemporary Drug Treatment of Advanced Heart Failure with Reduced Ejection Fraction. Drugs 2022; 82: 375-405.

Waterworth PD, Dunning J, Tolan M, Cozzi E, Langford G, Chavez G, White D, Wallwork J. Life-supporting pig-to-baboon heart xenotransplantation. J Heart Lung Transplant 1998; 17: 1201-7.

Wolf E, Kemter E, Klymiuk N, Reichart B. Genetically modified pigs as donors of cells, tissues, and organs for xenotransplantation. Anim Front 2019; 9: 13-20.

Wynyard S, Nathu D, Garkavenko O, Denner J, Elliott R. Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. Xenotransplantation 2014; 21: 309-23.

Yang B, Cui L, Perez-Enciso M, Traspov A, Crooijmans R, Zinovieva N, Schook LB, Archibald A, Gatphayak K, Knorr C, Triantafyllidis A, Alexandri P, Semiadi G, Hanotte O, Dias D, Dovc P, Uimari P, Iacolina L, Scandura M, Groenen MAM, Huang L, Megens HJ. Genome-wide SNP data unveils the globalization of domesticated pigs. Genet Sel Evol 2017; 49: 71.

Zaidi A, Schmoeckel M, Bhatti F, Waterworth P, Tolan M, Cozzi E, Chavez G, Langford G, Thiru S, Wallwork J, White D, Friend P. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584-90.

X. SUPPLEMENTARY INFORMATION

Table S1. Maintenance diet for Auckland Island pigs.

Ingredients	
Barley	31.5 %
Wheat	16.5 %
Soy husks	10.3 %
Wheat bran	10.0 %
Dried grass meal	10.0 %
Soy bean extract	8.5 %
Lignocellulose	3.5 %
Rapeseed extract	3.0 %
Vegetable oil	2.5 %
Oat husk meal	2.0 %
Mineral-vitamin-premix	2.2 %
Analyzed nutrient composition in original matter	
Dry matter	91.8 %
Crude protein	16.0 %
Crude fiber	11.4 %
Crude fat	2.3 %
Crude ash	5.9 %
N-free extracts	55.4 %
Calcium	7.8 g/kg
Phosphorus	5.3 g/kg
Zink	110 mg/kg
Copper	20 mg/kg

Table S2. Primer sequences for AO blood group and SLA genotyping.

Gene	Forward primer	Reverse primer	Amplicon length
AO blood group			
Blood group O	5′-CCATGGATAATCCTACCTGCTC-3′	5′-TAGGAACCATGAGGTTGCAG-3′	619 bp
Blood group A	5′-CGCCAGTCCTTCACCTACGAAC-3′	5′-CGGTTCCGAATCTCTGCGTG-3′	341 bp
SLA class 1			
*SLA-1*23*	5′-AAGCCCCGTTTCATCGCCGTC-3′	5′-ACTTCTGGAAGGTCCCATCCCCTG-3′	676 bp
SLA-2	5′-AGGGGCCCTCAAGCCATCCTCATTCTGC-3′	5′-AGTGTAGCTCCCTCCTTTTTCACCTG-3′	1020 bp
SLA-6	5′-ATCCCACTCGCTAAGATACCTCC-3′	5′-TCACAGTTCCAAGCACAGTGACCAC-3′	903 bp
SLA-11	5′-ACCCGAGCCCGAACACATTCTCTGC-3′	5′-TGGAGGTTCCCATCTCAGGGTG-3′	867 bp
SLA class 2			
DRA	5′-ATCACGTGATCATCCAGGCTGAG-3′	5′-TCAGTGGCGTTGCCTTTGCGCAC-3′	661 bp
DRB1	5′-TGTGTTTCTCCAGAGGCTTCTGGATGG-3′	5′-TCGTCAGGCTGGGGTGCTCCACTCGG-3′	621 bp
DQA	5′-TGATGAGCGCCTGTGGAGGTGAAGAC-3′	5′-AGTGGCTTATCCAGGCCCCAGTGC-3′	547 bp
DQB1	5′-TGCGGCTCCCCAGAGGCCTTTGGAC-3′	5′-AGGCTGGAGTGCTCCACGCGGCAGG-3′	616 bp

Table S3. Body dimensions of Auckland Island pigs.

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	SD	n	mean	SD	Age	Sex	Age*Sex
Body weight [kg]	8	16	5.37	1.41	10	5.17	1.14	**<0.0001**	0.8758	**<0.0001**
	12	16	11.51	2.23	10	10.89	1.93			
	16	11	20.14	3.83	9	18.13	2.19			
	22	16	29.06	3.52	10	26.63	3.81			
	30	14	42.64	6.11	5	36.20	3.63			
	40	13	58.15	6.17	5	46.90	3.01			
	155	1	110.00		1	134.00				
Crown rump length [cm]	8	16	39.44	3.78	10	39.20	3.26	**<0.0001**	**0.0011**	0.2593
	12	16	50.19	3.56	10	48.60	3.31			
	16	11	62.00	4.63	9	57.33	2.55			
	22	16	70.63	4.98	10	67.00	3.77			
	30	14	79.71	5.48	5	74.80	3.49			
	40	13	87.46	5.21	5	80.40	6.54			
	155	1	113.00		1	109.00				
Head length [cm]	8	16	14.25	1.18	10	14.00	1.76	**<0.0001**	**0.0079**	0.3706
	12	16	17.38	1.26	9	16.67	1.73			
	16	11	20.55	1.13	9	20.33	1.32			
	22	16	23.13	2.06	10	22.10	2.23			
	30	14	25.07	2.40	5	23.80	1.48			
	40	13	28.31	3.25	5	25.20	2.68			
	155	1	32.00		1	29.00				
Front leg height [cm]	8	16	29.25	3.36	10	28.40	2.88	**<0.0001**	**0.0004**	0.1931
	12	16	36.25	3.97	10	36.10	3.18			
	16	11	46.09	3.62	9	43.78	2.44			
	22	16	50.19	4.49	10	47.80	3.01			
	30	14	57.36	5.05	5	52.80	3.96			
	40	13	62.38	4.63	5	56.40	3.29			
	155	1	75.00		1	66.00				
Rear leg height [cm]	8	16	29.69	3.18	10	28.70	2.58	**<0.0001**	**0.0003**	0.2120
	12	16	37.19	3.54	10	36.30	2.71			
	16	11	46.45	4.13	9	44.56	2.19			
	22	16	50.56	4.34	10	48.90	2.51			
	30	14	57.07	4.87	5	52.60	3.91			
	40	13	62.00	4.53	5	56.00	2.92			
	155	1	75.00		1	66.00				
Chest circumference [cm]	8	16	40.19	3.89	10	40.40	3.63	**<0.0001**	0.1116	**0.0184**
	12	16	52.13	3.84	10	52.70	4.24			
	16	11	64.91	4.50	9	63.56	3.28			
	22	16	77.94	3.97	10	75.50	4.53			
	30	14	88.36	4.63	5	86.00	3.87			
	40	13	99.69	4.75	5	97.00	3.94			
	155	1	110.00		1	130.00				
Abdominal circumference [cm]	8	16	39.69	3.88	10	39.50	3.41	**<0.0001**	0.0867	**0.0230**
	12	16	50.81	3.33	10	52.20	4.87			
	16	11	64.55	4.32	9	64.67	3.28			
	22	16	77.25	4.93	10	74.00	4.19			
	30	14	87.71	5.82	5	85.00	5.15			
	40	13	96.54	5.17	5	95.00	3.81			
	155	1	105.00		1	125.00				

Table S4. Organ weights of Auckland Island pigs.

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	SD	n	mean	SD	Age	Sex	Age*Sex
Heart [g]	22	1	137.5		5	125.3	5.0	**<0.0001**	0.0562	0.4666
	40	14	172.0	42.8	4	139.6	21.8			
	155	1	422.0		1	331.3				
Lungs [g]	22	2	138.5	12.0	5	131.0	9.1	**<0.0001**	0.1667	0.6812
	40	15	177.2	40.4	4	140.5	31.5			
	155	1	352.0		1	310.3				
Liver [g]	22	2	660.0	28.3	5	604.0	65.4	**0.0045**	0.2142	0.6562
	40	15	960.6	275.2	4	692.1	147.1			
	155	1	1422.9		1	1241.1				
Kidney [g]*	22	2	77.7	1.5	5	58.8	6.4	**0.0002**	0.0619	0.7610
	40	15	102.2	33.3	4	63.2	6.2			
	155	1	193.3		1	159.6				
Pancreas [g]	22	2	64.7	4.8	5	57.7	8.1	**<0.0001**	0.4122	0.1244
	40	15	79.8	12.7	4	73.1	12.9			
	155	1	116.8		1	147.5				
Spleen [g]	22	2	76.3	7.1	5	99.9	30.5	0.3830	0.9502	0.3897
	40	15	188.4	125.8	4	91.9	47.4			
	155	1	169.4		1	231.0				

*Average weight of left and right kidney.

Table S5. Heart parameters of Auckland Island pigs.

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	95% CI	n	mean	95% CI	Age	Sex	Age*Sex
HR (bpm)	8	16	91.50	6.32	10	88.60	11.85	**<0.0001**	0.3101	0.3610
	12	16	89.56	7.15	10	88.60	7.83			
	16	11	72.82	8.60	9	77.00	3.80			
	22	16	67.75	6.84	10	59.40	5.27			
	30	14	63.79	4.94	5	70.80	11.25			
	40	14	68.92	6.43	5	58.20	6.83			
	155	1	78.00		1	63.00				
Teichholz (LAX)										
IVSd (mm)	8	16	5.79	0.31	10	5.67	0.36	**<0.0001**	**0.0038**	0.2863
	12	16	7.65	0.63	10	6.98	0.38			
	16	11	8.81	1.08	9	7.73	0.18			
	22	16	9.84	0.53	10	8.34	0.65			
	30	14	9.79	0.93	5	9.05	0.53			
	40	14	11.32	0.74	5	10.41	0.61			
	155	1	16.33		1	13.83				
LVIDd (mm)	8	16	22.77	1.48	10	23.37	1.75	**<0.0001**	0.9187	0.9000
	12	16	29.64	1.22	10	28.94	2.04			
	16	11	33.00	1.70	9	32.69	0.54			
	22	16	34.17	1.14	10	33.60	2.41			
	30	14	37.22	1.89	5	36.13	1.43			
	40	14	39.42	1.34	5	37.77	1.16			
	155	1	48.47		1	49.70				
LVPWd (mm)	8	16	4.10	0.26	10	4.36	0.43	**<0.0001**	0.8432	0.2362
	12	16	5.21	0.39	10	5.28	0.43			
	16	11	6.42	0.68	9	6.42	0.14			
	22	16	8.16	0.64	10	7.24	0.68			
	30	14	8.51	0.75	5	7.83	1.34			
	40	14	9.85	0.69	5	9.09	3.04			
	155	1	12.90		1	15.20				
IVSs (mm)	8	16	7.55	0.34	10	6.91	0.59	**<0.0001**	0.8499	**0.0307**
	12	16	10.17	1.02	10	9.54	0.77			
	16	11	11.24	1.24	9	11.59	0.20			
	22	16	14.31	0.80	10	12.11	0.83			
	30	14	14.13	0.99	5	15.61	4.34			
	40	14	16.28	1.01	5	15.49	2.00			
	155	1	19.47		1	20.97				
LVIDs (mm)	8	16	15.79	1.19	10	17.19	1.73	**<0.0001**	0.2963	0.1574
	12	16	20.07	0.95	10	19.09	1.35			
	16	11	21.97	1.21	9	21.18	0.38			
	22	16	21.01	0.77	10	20.98	1.63			
	30	14	22.60	1.24	5	20.49	3.64			
	40	14	24.23	0.98	5	21.73	1.96			
	155	1	32.73		1	29.93				
LVPWs (mm)	8	16	6.89	0.46	10	6.92	0.56	**<0.0001**	0.7311	0.0614
	12	16	8.68	0.49	10	8.64	0.64			
	16	11	10.48	0.55	9	10.58	0.24			
	22	16	11.82	0.86	10	10.65	0.95			
	30	14	12.62	1.00	5	10.12	1.36			
	40	14	15.12	0.87	5	14.38	3.16			
	155	1	20.57		1	20.80				
EF (%)	8	16	60.58	3.14	10	54.23	4.63	**<0.0001**	0.4842	**0.0004**
	12	16	62.23	1.84	10	64.70	1.50			
	16	11	63.30	1.78	9	65.78	0.63			
	22	16	69.83	1.34	10	68.87	1.15			
	30	14	70.67	1.00	5	68.60	1.18			
	40	14	69.38	1.07	5	70.53	0.98			
	155	1	60.67		1	70.00				
FS (%)	8	16	30.71	2.04	10	26.73	2.80	**<0.0001**	0.3848	**0.0009**
	12	16	32.29	1.28	10	33.97	1.25			
	16	11	33.42	1.29	9	35.15	0.49			
	22	16	38.46	1.11	10	37.70	0.73			
	30	14	39.38	0.83	5	37.67	1.15			
	40	14	38.50	0.85	5	39.20	1.30			
	155	1	32.33		1	39.33				

B-Mode and Doppler										
Ao (mm)	8	16	9.93	0.60	10	10.18	0.91	**<0.0001**	0.0527	0.1692
	12	16	12.95	0.72	10	12.86	0.73			
	16	11	16.00	1.15	9	14.99	0.29			
	22	16	17.24	0.93	10	16.30	0.87			
	30	14	18.54	1.08	5	17.10	1.23			
	40	14	19.54	1.20	5	18.46	1.61			
	155	1	31.60		1	26.40				
LA (mm)	8	16	14.49	0.79	10	14.64	1.37	**<0.0001**	**0.0480**	0.1725
	12	16	19.20	1.05	10	18.82	1.00			
	16	11	22.79	1.09	9	21.73	0.39			
	22	16	25.29	1.31	10	23.63	1.29			
	30	14	27.52	1.57	5	25.38	1.50			
	40	14	28.90	1.57	5	27.28	2.17			
	155	1	46.40		1	39.50				
LA/Ao	8	16	1.47	0.03	10	1.44	0.03	0.3098	0.5667	0.9301
	12	16	1.47	0.04	10	1.47	0.04			
	16	11	1.45	0.06	9	1.45	0.01			
	22	16	1.47	0.03	10	1.45	0.03			
	30	14	1.49	0.03	5	1.49	0.04			
	40	14	1.48	0.03	5	1.48	0.03			
	155	1	1.47		1	1.49				
PV (mm)	8	16	9.91	0.56	10	9.98	0.82	**<0.0001**	0.6054	0.3617
	12	16	12.90	0.71	10	12.61	0.56			
	16	11	15.97	0.86	9	15.38	0.24			
	22	16	17.59	0.99	10	16.62	0.72			
	30	14	18.82	1.34	5	17.66	1.40			
	40	14	19.51	1.14	5	20.60	2.55			
	155	1	29.40							
PV V_{max} (m/sec)	8	16	0.82	0.08	10	0.69	0.06	**0.0105**	0.8158	**0.0057**
	12	16	0.81	0.06	10	0.80	0.07			
	16	11	0.85	0.08	9	0.81	0.02			
	22	16	0.76	0.07	10	0.89	0.12			
	30	14	0.87	0.06	5	0.96	0.13			
	40	14	0.84	0.05	5	0.80	0.12			
	155	1	0.95							
LVOT V_{max} (m/sec)	8	16	1.11	0.14	10	1.03	0.19	**0.0107**	0.5172	0.3302
	12	16	1.25	0.08	10	1.12	0.08			
	16	11	1.17	0.09	9	1.20	0.03			
	22	16	1.17	0.07	10	1.24	0.08			
	30	14	1.27	0.07	5	1.28	0.14			
	40	14	1.35	0.08	5	1.23	0.13			
	155	1	1.15		1	1.15				
E-wave (m/sec)	8	16	0.77	0.07	10	0.72	0.07	0.8384	0.4500	0.5134
	12	16	0.87	0.03	10	0.85	0.07			
	16	11	0.82	0.04	9	0.92	0.03			
	22	16	0.78	0.05	10	0.82	0.07			
	30	14	0.80	0.06	5	0.85	0.03			
	40	14	0.86	0.05	5	0.82	0.09			
	155	1	0.82		1	0.85				
A-wave (m/sec)	8	16	0.41	0.05	10	0.33	0.03	**0.0002**	0.8185	0.1042
	12	16	0.43	0.04	10	0.43	0.05			
	16	11	0.45	0.04	9	0.50	0.03			
	22	16	0.46	0.04	10	0.48	0.08			
	30	14	0.44	0.04	5	0.50	0.05			
	40	14	0.47	0.04	5	0.44	0.08			
	155	1	0.57		1	0.52				

Abbreviations: Ao = aortic valve diameter (mm); A-wave = A-Wave of mitral inflow in PW doppler; CI = confidence interval; EF =ejection fraction (%); E-wave = E-Wave of mitral inflow in PW doppler; FS = fractional shortening; HR = heart rate; IVSd = interventricular septum thickness in diastole (mm); IVSs = interventricular septum thickness in systole (mm); LA = left atrial diameter in SAX; LA/Ao = relation of left atrial diameter to aortic diameter; LVIDd = left ventricular diameter in diastole; LVIDs = left ventricular diameter in systole; LVOT V_{max} = bloodflow of left ventricular outflow tract in CW doppler; LVPWd = left ventricular parietal wall thickness in diastole; LVPWs = left ventricular parietal wall thickness in systole; PV = pulmonary valve diameter; PV V_{max} = bloodflow of pulmonary valve in PW doppler.

Table S6. Clinical-chemical and hematological parameters of Auckland Island pigs.

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	95% CI	n	mean	95% CI	Age	Sex	Age*Sex
Clinical chemistry										
Glucose [mmol/L]	12	16	5.54	0.23	10	5.53	0.40	0.8111	0.6256	0.4598
	22	16	5.24	0.43	10	5.97	1.30			
	40	14	5.48	0.93	5	5.40	1.01			
	155	1	4.70		1	6.80				
Urea [mmol/L]	12	16	4.12	0.73	10	4.77	0.59	0.6548	0.7333	0.1347
	22	16	4.78	0.47	10	4.58	0.70			
	40	14	4.27	0.90	5	5.82	1.47			
	155	1	5.90		1	3.50				
Creatinine [µmol/L]	12	16	42.99	4.89	10	36.86	6.31	**<0.0001**	**0.0001**	**0.0296**
	22	16	65.52	6.52	10	59.78	5.11			
	40	14	83.44	8.80	5	70.72	9.89			
	155	1	157.44		1	99.95				
Total protein [g/L]	12	16	60.80	1.67	10	60.85	3.15	**<0.0001**	0.5451	0.4166
	22	16	61.23	3.73	10	59.73	4.21			
	40	14	67.14	2.39	5	70.40	5.09			
	155	1	84.90		1	75.40				
Albumin [g/L]	12	16	42.89	1.75	10	42.62	2.53	0.0947	0.8225	0.3381
	22	16	45.13	1.45	10	42.12	3.59			
	40	14	45.15	6.38	5	50.12	4.49			
	155	1	46.20		1	44.80				
Total bilirubin [µmol/L]	12	16	3.15	1.68	10	2.33	1.13	**0.0040**	0.8219	0.9734
	22	16	0.80	0.27	10	0.71	0.20			
	40	14	1.66	0.61	5	1.08	0.62			
	155	1	1.25		1	1.60				
ALP [U/L]	12	16	293.31	44.26	10	265.60	42.78	**<0.0001**	0.8435	0.9361
	22	16	229.06	24.28	10	214.80	26.56			
	40	14	178.21	25.39	5	189.20	20.27			
	155	1	38.00		1	33.00				
AST [U/L]	12	16	50.35	23.25	10	78.26	44.02	0.1881	0.9899	0.7723
	22	16	116.67	110.57	10	183.81	103.21			
	40	14	38.95	13.20	5	29.82	15.81			
	155	1	41.90		1	14.40				
GGT [U/L]	12	16	29.95	4.45	10	29.68	4.43	**0.0014**	0.8567	0.7769
	22	16	34.87	2.57	10	34.62	4.21			
	40	14	40.48	5.64	5	35.60	3.23			
	155	1	41.80		1	45.00				
CK [U/L]	12	16	2310.50	1238.47	10	4307.50	3144.01	0.2004	0.9188	0.9648
	22	16	6521.27	8524.52	10	8392.00	6118.59			
	40	14	2321.64	1193.19	5	1389.20	1233.16			
	155	1	1805.00		1	900.00				

Abbreviations: ALP = alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; CK = creatine kinase; GGT = gamma glutamyltransferase.

Table S6. Clinical-chemical and hematological parameters of Auckland Island pigs (continued).

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	95% CI	n	mean	95% CI	Age	Sex	Age*Sex
Electrolytes										
Na [mmol/L]	12 22 40 155	16 16 14 1	133.00 141.69 137.92 138.00	1.37 4.11 2.96	10 10 5 1	131.10 140.70 137.20 139.00	2.12 4.46 4.78	0.0668	0.8079	0.8036
K [mmol/L]	12 22 40 155	16 16 14 1	4.44 5.32 4.77 4.28	0.37 0.36 0.46	10 10 5 1	4.96 5.26 4.67 5.19	0.44 0.47 0.56	0.0798	0.4881	0.5678
Ca [mmol/L]	12 22 40 155	16 16 14 1	2.52 2.14 2.47 2.30	0,05 0,21 0,08	10 10 5 1	2.52 2.22 2.42 2.43	0.07 0.23 0.08	0.8183	0.7479	0.7736
Mg [mmol/L]	12 22 40 155	16 16 14 1	1.06 1.04 1.10 1.20	0.06 0.10 0.08	10 10 5 1	1.11 1.03 1.08 0.85	0.07 0.10 0.05	0.6453	0.1641	0.3946
Cl [mmol/L]	12 22 40 155	16 16 14 1	91.08 100.29 94.16 98.60	1.62 4.56 2.24	10 10 5 1	89.22 98.23 65.74 101.40	1.94 5.27 5.75	**<0.0001**	0.9247	0.7946
Phosphorus [mmol/L]	12 22 40 155	16 16 14 1	2.78 2.60 2.17 1.80	0.18 0.18 0.16	10 10 5 1	2.96 2.53 2.04 1.80	0.31 0.17 0.21	**<0.0001**	0.9094	0.8476
Fe [µmol/L]	12 22 40 155	16 16 14 1	17.43 25.08 25.09 16.96	3.38 2.31 5.22	10 10 5 1	19.59 31.87 30.99 22.21	2.86 3.06 7.67	**0.0002**	0.2188	0.4025

Abbreviations: CI = confidence interval.

Table S6. Clinical-chemical and hematological parameters of Auckland Island pigs (continued).

Parameter	Age [wk]	Males			Females			Analysis of variance		
		n	mean	95% CI	n	mean	95% CI	Age	Sex	Age*Sex
Hematology										
Leukocytes [G/L]	12 22 40 155	16 16 14 1	10.17 13.14 10.70 5.84	1.69 2.00 1.05	10 10 5 1	11.74 13.70 10.89 12.24	3.25 1.03 2.37	**0.0252**	0.1275	0.6578
Erythrocytes [T/L]	12 22 40 155	16 16 14 1	6.97 7.78 7.01 6.46	0.35 0.46 0.47	10 10 5 1	6.93 7.87 6.98 3.78	0.38 0.55 0.32	**<0.0001**	0.0863	0.1315
Hemoglobin [g/L]	12 22 40 155	16 16 14 1	115.8 125.9 123.8 123.0	6.5 8.3 8.0	10 10 5 1	117.7 125.7 125.6 71.0	7.4 10.7 9.0	**0.0085**	0.0529	0.0965
Hematocrit [%]	12 22 40 155	16 16 14 1	40.84 44.77 45.84 46.03	2.70 2.87 2.80	10 10 5 1	42.44 45.74 45.60 48.99	2.62 3.74 3.18	**0.0087**	0.1174	0.1126
Thrombocytes[G/L]	12 22 40 155	16 16 14 1	342.81 232.50 227.46 150.00	48.19 52.95 34.75	10 10 5 1	269.30 233.88 177.00 179.00	80.89 31.92 63.25	**0.0002**	0.1581	0.8957
Cell differentiation										
Rod nucleated neutrophils [%]	12 22 40 155	16 16 14 1	14.13 9.93 5.07 2.00	4.11 4.32 1.42	10 10 5 1	9.20 6.60 4.20 1.00	3.06 4.16 3.58	**0.0043**	0.3114	0.6620
Segmental nucleated neutrophils [%]	12 22 40 155	16 16 14 1	38.75 26.93 44.86 46.00	5.84 5.45 7.93	10 10 5 1	32.50 22.30 33.60 59.00	6.25 2.98 14.07	**0.0002**	0.7082	0.5260
Lymphocytes [%]	12 22 40 155	16 16 14 1	41.69 57.73 42.93 43.00	5.18 6.63 7.79	10 10 5 1	50.70 63.50 57.20 38.00	4.19 6.50 15.65	**0.0005**	0.2347	0.6419
Monocytes [%]	12 22 40 155	16 16 14 1	4.81 4.47 5.79 6.00	1.69 1.06 1.43	10 10 5 1	7.40 5.90 2.00 1.00	2.03 2.18 1.07	0.1608	0.2750	**0.0172**
Eosinophilic granulocytes [%]	12 22 40 155	16 16 14 1	0.31 0.93 1.00 0.00	0.30 1.06 1.30	10 10 5 1	0.00 1.70 2.20 1.00	0.00 1.28 1.69	**0.0333**	0.3358	0.5428
Basophilic granulocytes [%]	12 22 40 155	16 16 14 1	0.31 0.00 0.21 2.00	0.30 0.00 0.30	10 10 5 1	0.10 0.00 0.40 0.00	0.20 0.00 0.78	**0.0152**	**0.0084**	**0.0197**

Abbreviations: CI = confidence interval; G = giga (10^9); T = tera (10^{12}).

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