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REVIEW ARTICLE

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On the way (my way) to clinical xenogeneic heart transplantation. Presented at the 15th biannual IXA meeting, Munich, October 11, 2019

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1 | PRECLINICAL AND CLINICAL CONCORDANT HEART XENOTRANSPLANTATION

The first heart replacement in humans was a xenograft. On January 23, 1964, James Hardy (1918-2003) from Mississippi, USA, removed the heart of a dying 68-year-old adult and replaced it with the organ of a small chimpanzee. After 90 minutes, the transplant stopped beating, because it was of course too small.[1] Hardy was heavily criticized by both the public and his medical peers—partly unfair since he and his team had prepared the intervention carefully over years; he should, however, have selected a better first recipient.

Next was Christiaan Barnard (1922-2001) from Cape Town, South Africa. In 1973, he used a baboon and a chimpanzee heart in two patients who were in desperate need of a new organ (in those apartheid times, white recipients were only allowed transplants from the same skin color—at that time a cause of organ shortage in South Africa). Due to the size mismatches of the organs (recipients vs donors), Barnard wisely used his piggyback-technique, leaving the patient's heart in place and using the small transplants as (possibly temporary) support until a human organ would eventually be available. However, both recipients died early: the baboon organ failed within hours, and the recipient of the chimpanzee heart died after 4 days.[2,3]

The interventions should have been more successful. A convincing explanation was not given; the two interventions also created a great stir within the community of the university, a reaction which Barnard left unmentioned.

At the Ludwig-Maximilians University in Munich, Walter Brendel (1922-1989) became Head of the Department of Experimental Surgery (now the "Walter Brendel Center for Experimental Medicine"). During most of the 1960s till the first half of the 1980s, xenotransplantation remained one of his main interests—and that of his consultant and chief investigator Claus Hammer (1940-2015). The latter characterized preformed natural antibodies (PNABs)[4] in sera of 48 species from seven zoological orders and investigated more than 8300 combinations of serum samples and antigens of 111 individuals. Among others, his key finding was that PNABs were absent or low between the species within a zoological family (concordant systems) such as domestic dogs, foxes and dingos; domestic cats, lions, and tigers; and man and old world monkeys. Heterotopic intrathoracic fox-to-dog heart transplants remained beating (but not working) for an average of 20 days using cyclosporine and cortisone.[5,6]

In contrast, PNABs were augmented across divergent species (discordant systems); corresponding experiments yielded discouraging results since the grafts never functioned longer than a few hours, in spite of aggressive additional treatments with either lymphatic drainage or plasmapheresis.[7]

Both Brendel and Hammer were therefore convinced "that concordant non-human primate to human xenotransplantation would ultimately become a clinical reality," but in their opinion, discordant procedures would remain unrealistic. They did, however, concede that if it ever became feasible it would revolutionize medicine.[8]

At approximately the same time, Leonard Bailey (1942-2019) from Loma Linda, USA had transplanted Baby Fae, a premature newborn patient with hypoplastic left heart syndrome. She barely survived

on her right heart only which was connected to the (left) systemic circulation via a huge atrial septum defect and a patent Botalli duct; the left heart, after which the syndrome is named, was minute as were the mitral and aortic valves, the ascending aorta and the arch. At that time, heart transplantation, including the replacement of the whole aortic arch, was considered the only option (it is important to mention that newborns with such a cardiac malformation have usually no further congenital lesions; in particular the brain is well developed). Unable to get a human donor in time, Bailey transplanted a histocompatible baboon heart on October 10, 1984. Baby Fae, who was blood group O, succumbed after three weeks to disseminated hemagglutination: The baboon had been blood group incompatible; all six donors which were available to Bailey were blood group AB.[9,10]

Bailey never did another baboon-to-human transplant. As a matter of fact, he did not have to, since the reaction to this one and only xenogeneic intervention was unexpected: suddenly Bailey received human donors of suitable sizes from all over North America, and his pediatric cardiac transplant program took the lead worldwide.[11]

In the second half of the 1980s, a similar program was set up at the University of Cape Town,Medical School (following the advice of Brendel and Hammer; the latter and Bailey were guests of a Capetonian meeting commemorating the 20th anniversary of the first human heart transplant in 1987; Figure 1). An experimental program was set up, transplanting green vervet monkey hearts heterotopically into the neck of recipient baboons.[12,13] Various immunosuppressive regimens were tested; the combination of anti–thymocyte-globulin, methylprednisolone, azathioprine, and cyclosporine proved best, with the hearts beating (but not working) for up to 83 days.

The results of the experimental study quickly became a clinical reality when two babies were referred to the Department of Cardiac Surgery in the Red Cross Children's Hospital, both presenting with hypoplastic left heart syndromes. After the parents agreed to the xenotransplantation (actually not a very difficult discussion), two captive-bred, blood group-compatible baboons with known microorganisms were ordered from the South African Medical Research Council.[14] The preparations for the transplants came to a sudden halt when one morning both donors were found dead in their cages. No causes were identified, but the message was clear: The University and the South African society did not want concordant xenotransplantation.

To finish the story, both potential recipients died soon thereafter, one from pneumonia and the other from cerebral bleeding.

2 | COMMENCING PRECLINICAL DISCORDANT CARDIAC XENOTRANSPLANTATION IN NON-HUMAN PRIMATES

At the end of his Capetonian time, DKC Cooper conducted some stimulating experiments: after first perfusing porcine wild-type kidneys with recipient baboon blood, and thereby removing preformed xenogeneic antibodies, subsequent wild-type porcine heart transplants avoided hyperacute rejection and beat significantly longer when compared to control; the porcine hearts were connected to the neck vessels of the baboons,[1,2,4,15] and cyclosporine and methylprednisolone were given. These experiments were a proof of principle that discordant cardiac xenotransplantation would be possible when natural preformed antibodies could be deleted.

In the early 1990s, David White (1946-2017) started generating transgenic pigs expressing the human complement regulatory protein hDAF (decay-accelerating factor, hCD55).[18] His first animal, Astrid, was born in December 1992 (David White always claimed it was the 25th). Not much later, he invited Claus Hammer and his Munich group to Cambridge and Immutran/Novartis for a week's cooperation to perfuse extracorporal hearts (livers and kidneys) of five DAF-animals with human blood (with three of Hammer's perfusion machines; the German researchers served also as blood donors).[8] The 15 experiments documented the prevention of hyperacute rejections.[18-20] In 1997, The Lancet declared these results the ground-breaking achievement of the year.

FIGURE 1 Participants of the meeting commemorating the 20th anniversary of the first human heart transplantation, Cape Town, 1987; Leonhard Bailey first row, second from the right; Claus Hammer in the middle, second row

Everyone in the field became excited and long-term results were achieved in the abdominal heterotopic position (the heart transplants were attached to the abdominal infrarenal aorta, to the inferior vena cava; [21] reviewed in[15]). Fundamental ethical issues were thoroughly discussed[22,23] and in 2000, an Advisory Committee of the International Society for Heart and Lung Transplantation was set up, which proposed its now famous efficacy recommendations (not guidelines) for future clinical cardiac applications: 60% success in a consistent group of at least 10 animals. Three months survival was suggested for a minimum, but longer periods should be aimed for.[24]

And the xenogeneic heart transplantation technique had to be life supporting. These were ambitious targets in times when median survival after orthotopic procedures was measured less than one month ([21] also,[24,25] the first author M. Schmoeckel from Munich University did that study[21] during his stay at Immutran/Novartis; the cardiac surgeon J. Wallwork and D. White were the senior authors).

The importance of porcine galactose-α1,3-galactose (Gal) was recognized: Non-antigenic Gal-polymers were given to bind the respective natural Gal-antibodies. In the heterotopic abdominal position, the grafts beat in baboons for a record median of 96 days (range 15-137 days).[26] These findings would have fulfilled the targets set by the 2000 Advisory Board only five years before—if the results had been achieved in a life-supporting system.

Homozygous Gal-free modified pigs (GGTA1-KO) became available in the early 2000s.[27] Not long thereafter, the first results using GGTA1-KO porcine hearts (again in the heterotopic abdominal position) were reported by the Boston group which was led by DH Sachs and DKC Cooper[28]: using immunosuppression and co-stimulation blockade (anti-CD154 ab), the median survival time of the grafts amounted to 78 days (range 16-179 days). Myocardial histology revealed signs of thrombotic microangiopathy.

In these times, however, the future of xenotransplantation looked all of a sudden grave: C. Patience, Y. Takeuchi, and RA Weiss published their influential study on Porcine Endogenous Retroviruses (PERVs) and potential infectious risks after xenogeneic procedures.[29] Severe ethical questions were raised and even a moratorium of xenogeneic procedures was discussed; F. Bach summarized: The benefits (and risks) of the treatment of a terminally ill individual must be weighed against the collective risk for a whole society.[30] As a consequence, Immutran/Novartis was closed, as was the William J. von Liebig defined pathogen free (DPF) unit in Rochester next to the Mayo Clinic. Well, xenotransplantation survived and 20 years thereafter, RA Weiss summarized without regrets[31]: "If we had not investigated PERVs in the 1990s, we would not have (eg) pigs, free from known infectious (microorganisms) today."

During these last two decades, safety issues remained one of the major topics in the field. No PERV-infections were seen in humans.[32,33]

It is therefore remarkable that these were the times when in 1998 preclinical discordant xenotransplantation (using non-human primates) commenced in Germany, first supported by the Bavarian, then the German Research Foundation. The Consortium has now three pillars: Munich (Ludwig-Maximilians and Technical Universities), Hannover (Medical School), and Dresden (Technical University). While in Germany, all universities are funded by the respective States, it was wise to attach four Federal (German wide) Institutions: Robert-Koch (Berlin), Paul-Ehrlich (Langen), Friederich-Löffler (Mariensee), and the German Primate Center (Göttingen). Together, this Collaborative Research Center is a consortium of immunologists, bioengineers, virologists, primatologists, ethicists, legal authorities, and clinicians.

In the first decade of the 21st century, preclinical xenogeneic orthotopic (life supporting) heart transplantation was the focus of our Munich cardiosurgical team. The results were, however, inconsistent and unpredictable—as they were everywhere else [25,34-41]: the survival rates in non-human primates ranged from 1 to 57 days, with an unacceptably high 40%-60% perioperative mortality, although clinically approved heart preservation techniques were applied (after allogeneic procedures, primary failure rates are four to six times lower). G. Byrne and C. McGregor termed the phenomenon "Perioperative Cardiac Xenograft Dysfunction" (PCXD).[21,42] They concluded that PCXD was not a hyperacute rejection reaction, but resembled more ischemic reperfusion injury or the "old-fashioned" cardiac stunning of the early days of heart surgery, when effective cardioplegic techniques were not available.

3 | INTRATHORACIC (LIFE SUPPORTING) HETEROTOPIC XENOTRANSPLANTATION

Until 2007, our team in Munich exclusively used hDAF-expressing donor animals from the previous Immutran/Novartis farm, and the non-antigenic Gal-polymer GAS914 was given together with standard immunosuppression.[40] During that time, two organs from hCD46 transgenic animals were also transplanted orthotopically into baboons: The live donors remarkably passed the intercontinental borders between North America and Germany without any delay (the animals originated from the William J. von Liebig DPF-unit in Rochester, Mn.).

After the 9th IXA meeting in Minneapolis, 2007, we had access to double genetically modified (GGTA1-KO, hCD46-tg) pigs from D. Ayares (Revivicor, Blacksburg, Va.). In cooperation with E. Wolf and his team (Molecular Animal Breeding and Biotechnology, Gene-Center; Center for Innovative Medical Models, CiMM, both LMU, Munich), human thrombomodulin (hTBM) was added.[43] This was the time when intrathoracic heterotopic heart transplantation was contemplated, a preclinical model combining the prerequisite of a life-supporting technique with the advantages of two hearts which to various extents contributed to the combined (total) cardiac output: when, for example, immediately after surgery the recipient heart may still have to produce the major share in case a transplanted organ is not able to perform adequately due to ischemia/reperfusion damage. The transplants will then recover after a few days and take over the brunt of the (combined) left-sided ventricular output.

Intrathoracic heterotopic heart transplantation was clinically introduced by Christiaan Barnard 50 years ago,[44,45] whereby the

donor organ is placed within the right chest and next to the recipient heart. Four anastomoses are needed: between the left and right atria, the end-to-side connections of the aortae, and the pulmonary arteries (with an interposition vascular graft). Clinical hemodynamic and echocardiographic measurements proved that the transplanted heart made up on average 73% of the total cardiac output.[46] Long-term results were good with 1, 2, and 5-year(s) survival rates of 63%, 54%, and 43% (therefore slightly inferior when compared to orthotopic procedures.[47]

Twenty-one consecutive experiments were carried out in baboons between 2009 and 2013.[48] Using Bretschneider's crystalloid HTK cardioplegic solution,[49,50] PCXD was not seen and in 19/21 cases, the recipients came off cardio-pulmonary bypass without difficulties (technical failures were the cause of death of the remaining two). Myeloablative induction therapy like that used for myeloma patients (anti-CD20ab, Cyclophosphamid, ± Bortezomib)[51] was given and immunoglobulin-apheresis added immediately before surgery. Maintenance immunosuppression consisted of anti-CD20ab, Tacrolimus, MMF, and steroids. In a second group, thoracoabdominal lymphoid irradiation with six Grays was added; co-stimulation blockade was not available.

Overall mean survival (excluding four technical failures) was 21.7 ± 6 days, with the longest lasting for 50 days. Echocardiography showed that mitral and aortic valves opened and closed, proving the contribution of the transplant to the cardiac output (and of course, there were additional arterial pressure curves on the monitor). Bacterial and fungal infections were common (at that time, we had to share our operative room with other groups). In the longer-term survivors, an excessive graft overgrowth of more than 200% within one month was seen, a phenomenon we could not explain. These transplants compressed more or less the whole right lung; as a consequence, the baboons had to remain in an oxygen tent which enclosed their cages.

In one case, the donor heart compressed most of the recipient's left atrium causing finally recalcitrant lung edema (Figure 2). In this context, it should be mentioned that the signs of overgrowth observed in our baboon experiments had never been seen in clinical practice ([47], personal experience).

Taken together, we concluded that intrathoracic heterotopic xenogeneic heart transplantation would be a useful clinical tool provided an immunosuppression was less toxic. The technique may offer advantages, especially under the circumstances of a first clinical application followed by an unexpected graft failure. The recipient's own heart could then serve as a back-up until a new transplant—human or preferably porcine—was available.

Intrathoracic heterotopic xenogeneic heart transplantation would also be a good solution in patients with severe pulmonary hypertension. Under these circumstances, the patient's own (adapted) right ventricle would support the respective (not adopted to high pressures) donor chamber.[46]

Right at that time, M. Mohiuddin (NIH, Bethesda, USA) published his first results after heterotopic abdominal heart xenotransplantation with the same genetically modified GGTA1-KO, hCD46-tg (in a last group plus hTBM) Revivicor donor hearts,[52] using a chimeric 2C10R4 anti-CD40 antibody, which was available to us after the 11th IXA meeting, 2011, in Miami Beach. Two successful intrathoracic heterotopic transplants were done. Thereafter we were however forced to change our plans: To fulfill the new European Directive on the Protection of Animals used for Scientific Purposes and a decision of local Upper Bavarian Authorities, we had to renovate our facilities. In a new operative room, exclusively dedicated to cardiac xenotransplantation, orthotopic procedures had to be done by order.

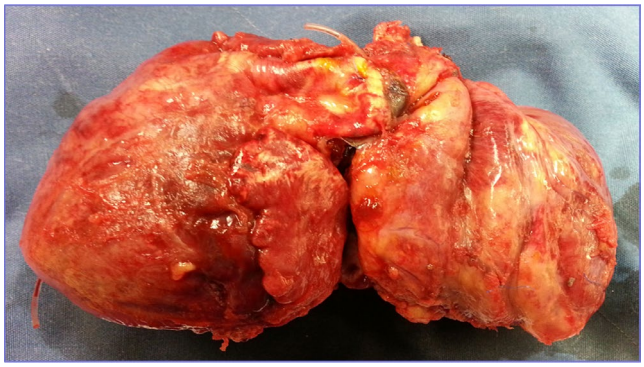

FIGURE 2 Post-mortem frontal view after heterotopic intrathoracic pig-to-baboon heart transplantation (porcine transplant to the right of the recipient organ; pulmonary end-to-side anastomosis with interposition graft shown): note transplant overgrowth on post-operative day 19, it was initially smaller than the recipient heart (from[48]; at that time we could not explain the phenomenon)

4 | PRECLINICAL ORTHOTOPIC CARDIAC XENOTRANSPLANTATION

In the meantime, M. Mohiuddin had published his milestone achievement after heterotopic abdominal heart xenotransplantation,[53] using high dose anti-CD40 antibody. In a last group (n = 5), a median graft survival of 298 days was achieved, with a maximum of 945 days.

Our 14 orthotopic experiments, using an immunosuppression protocol slightly changed from Mohiuddin's, were performed between 2015 and 2018.[54] Unfortunately, group I (n = 5) came to a quick end: using clinically approved crystalloid cardioplegic solutions, survival times were one day (n = 3), 3 and 30 days; severe systolic pump failure was diagnosed in 4 cases (low cardiac output despite high dosages of catecholamines, the so-called "Perioperative Cardiac Xenograft Dysfunction" (PCXD)).

PCXD was eliminated in group II (n = 4) after we introduced "Non-ischemic Porcine Heart Preservation." In cooperation with S. Steen's group from Lund University, Sweden, the grafts were immediately perfused with a 8°C hyperoncotic cardioplegic solution containing erythrocytes, nutrition, and hormones[55] (Figure 3); perfusion was intermittently continued even during implantation. The survival times were 4 (technical failure), 18, 27, and 40 days. The three long-term survivors succumbed to rapidly developing graft overgrowth with signs of terminal diastolic pump failure and consecutive liver

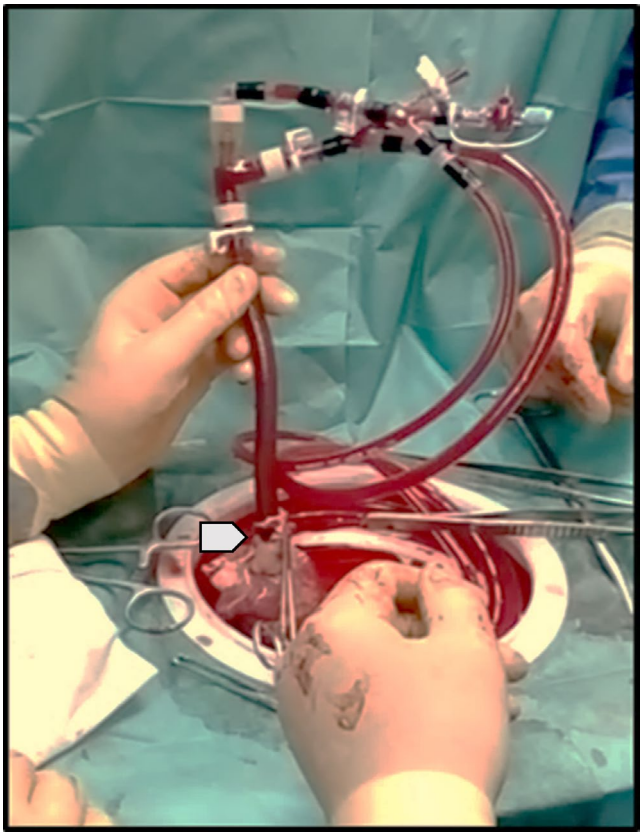

FIGURE 3 Genetically modified porcine heart with the perfusion cannula in the ascending aorta (arrow)

damage. Detrimental xenograft overgrowth was thought to be intrinsic, that is, genetically determined (changes as for an ultimately 200 kg German Landrace pig, lethal in the small chest of a baboon recipient). Similar findings have been described in pig-to-baboon kidney transplantation[56] (see also Figure 2).

In the last group III (n = 5), cardiac overgrowth was successfully counteracted by:

1. decreasing the blood pressure of the baboons (pigs have lower blood pressures[57]),
2. early weaning from Cortisone, which can cause hypertrophic cardiomyopathy in early human life,[58] and
3. treatment with the Sirolimus prodrug Temsirolimus to inhibit growth-hormones by blocking mTOR-kinases.[59]

Altogether, one animal was lost to recalcitrant pleural effusion due to thoracic lymph duct occlusion. All others survived long term: two recipients for three months, two others for 182 and 195 days; all four were euthanized in good general condition.

In the meantime, the group was completed according to the efficacy requirements of the Advisory Board of the ISHLT [24,60]: Four additional cases were added of whom two reached the 3 months mark, and another two succumbed early to generalized porcine cytomegalovirus (PCMV) infections; PCMV will be avoided in the future by early weaning [61] and finally by animal selection.

Taken together, six out of eight consecutive baboons survived orthotopic xeno-heart transplantation for at least three months, exceeding the threshold set by the Advisory Board.

5 | OUTLOOK

What remains to be done before entering the clinical scenario of a pilot study? According to a first scientific advice by the Innovation Office of the Paul-Ehrlich Institute (the German representative of the European Medicines Agency, EMA), regulatory requirements will not be unsurmountable—but they will, however, take time. Additional gene modifications might be helpful, like hCD47[62] or PD-L1[63]; the question of the ideal co-stimulation blockade will have to be answered.[64,65] And last but not least, the size/growth of a donor organ will matter, especially in smaller recipients.[54,66]

Dealing with the PERV-issue—two solutions are now available and widely accepted:

1. selecting PERV-C-free donor animals to avoid A/C recombinants which have a high replication rate in infected human cells[67-69]; also the virus load of PERV A and B should be low.
2. generation of healthy pigs without PERVs using CRISPR/Cas technology, as demonstrated by eGenesis, Boston.[70,71]

Since both strategies are clinically acceptable, cost, and affordability might be a crucial point.

And finally the advice of Weiss[31]: "While the promise of clinical xeno-transplantation once again looks very bright, disregard to infectious risks, we know we must not relapse into complacency."

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