Long-term outcome of homografts related to donor characteristics and tissue processing

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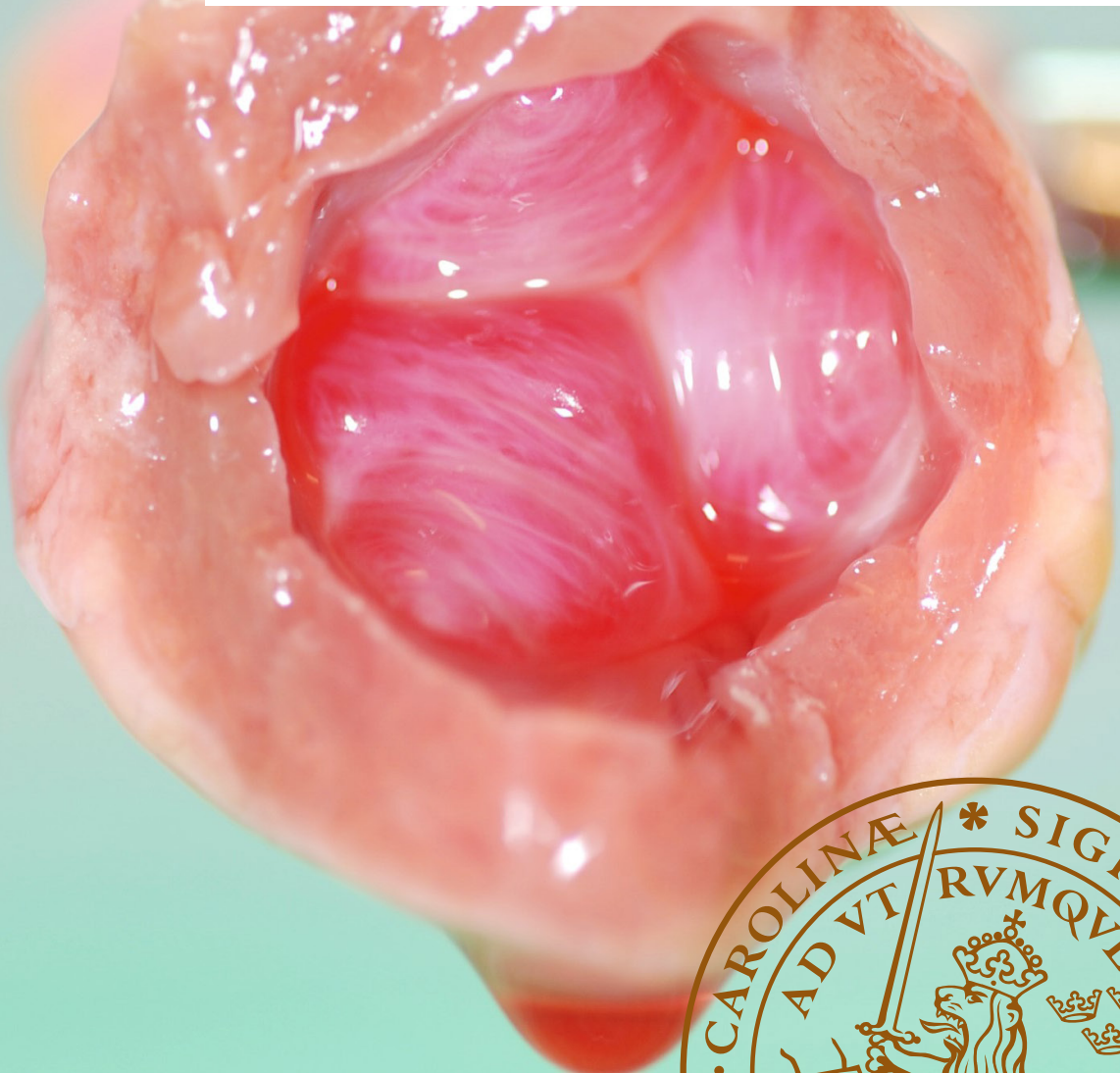

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Ida von Konow

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 26th of January at 09.00 in Segerfalksalen, BMC, Lund

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Abstract

Background. The homograft is an excellent conduit for right ventricular outflow tract (RVOT) reconstruction, but it has limited durability and availability.
Aims: I-III: Evaluate donor, homograft, and recipient characteristics in relation to long-term outcomes after RVOT reconstruction. **IV:** Evaluate mechanical properties of homografts after different decontamination intervals. **V:** Evaluate homograft ultrastructures after different decontamination intervals.
Methods. Studies I-III were of a retrospective design and included homografts implanted in the RVOT. In study I, 297 homografts were included and in studies II and III, 509 homografts were included. The follow-up was 0-24 years. Risk factors for reintervention were analyzed with the Kaplan-Meier method for study I and Cox proportional hazard regression for studies I-III. Study IV included 10 mechanically tested homografts after four different decontamination times. Elastic modulus, yield stress and energy at yield stress were calculated and compared between short (two to four days) and prolonged decontamination time (seven to nine, 28-30 or 60-62 days). Differences were analyzed with the Wilcoxon signed rank test. Study V included 32 homografts with biopsies collected at preparation (day 0) and after one, two, three, four, seven, 14, 21, 28 and 60 days of decontamination. Biopsies were studied using light microscopy (LM) and transmission electron microscopy (TEM) to evaluate cells, elastin, and collagen. Differences were compared between day 0 and a prolonged time (1-60 days) and analyzed with a Wilcoxon signed rank test.
Results: I: Aortic homografts, young donor age and an ischemic time of 1-24 hours (compared to 0 hours) were identified as risk factors for early reintervention. There was no difference when extending the ischemic time to 48 hours compared to 24 hours. II: Structural impairment of homografts was not a risk factor for early reintervention. III: Microbiological contamination before antibiotic decontamination during processing was not a risk factor for early reintervention or the development of endocarditis. IV: Elastic modulus and yield stress were significantly higher at two to four days compared to prolonged time. There were no significant differences in energy at yield stress at different times. V: LM found a decrease in cell count after 60 days of decontamination. TEM found signs of cell degeneration after three days and beyond, signs of elastin degeneration after 21 days and signs of collagen degeneration after 28 days of decontamination.
Conclusions: I: There is no harm in choosing a homograft from an older donor. It is possible to prolong the ischemic time to 48 hours without affecting the long-term outcome of homografts. II: Current guidelines on structural impairments of the homograft are acceptable. III: There is no harm in using homografts that were contaminated before decontamination if follow-up cultures are negative. IV: There could be some deterioration of the homograft resulting in decreased values of Elastic modulus and yield stress with a prolonged decontamination time. V: The earliest signs of elastin and collagen degeneration could be found after 21 and 28 days of antibiotic decontamination. Decontamination in a cold environment could be prolonged to at least 14 days without affecting homograft quality.

Key words: Homograft, Right ventricular outflow tract, Congenital heart surgery, Tissue banks

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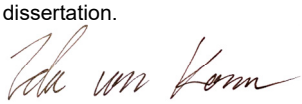
Signature:  Date: 2023-12-08

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Abbreviations

ACHD – adults with congenital heart disease
AV – aortic valve
CI – confidence interval
DH – decellularized homograft
EDQM – European directorate for the quality of medicine and health care
HLA – human leukocyte antigen
HR – hazard ratio
IE – infective endocarditis
IQR – interquartile range
LM – light microscopy
LVOT – left ventricular outflow tract
MOD – multi-organ donors
NHBD – non-heart-beating donor
PA – pulmonary atresia
PI – pulmonary insufficiency
PS – pulmonary stenosis
PV – pulmonary valve
RV – right ventricle
RVOT – right ventricular outflow tract
SD – standard deviation
TCPC – total cavopulmonary connection
TEM – transmission electron microscopy
TPVI – transcatheter pulmonary valve implantation

Abstract

Background. The homograft is an excellent conduit for right ventricular outflow tract (RVOT) reconstruction, but it has limited durability and availability.

Aims. This thesis aims to evaluate different aspects of homograft processing to maximize the use of available homografts without compromising on quality.

I. To evaluate donor, homograft, and recipient characteristics in relation to long-term outcomes after RVOT reconstruction with a homograft.
II. To analyze the impact of structural impairment of the homograft on long-term outcomes after RVOT reconstruction.
III. To assess the impact of microbiological and fungal contamination of homografts before antibiotic decontamination during processing, and the effect of such contamination on long-term outcome and risk of endocarditis after RVOT reconstruction.
IV. To calculate the differences in mechanical properties of homografts after prolonged decontamination intervals.
V. To study cells, elastin, and collagen of the homograft after prolonged decontamination intervals, and determine if it is possible to find a specific time when degeneration can be detected.

Methods. Studies I-III were of a retrospective design and included homografts implanted in the RVOT. In study I, 297 homografts were included. In studies II-III, 509 homografts were included. The follow-up was 0-24 years. Risk factors for reintervention were analyzed with the Kaplan-Meier method for study I and Cox proportional hazard regression for studies I-III. Study IV included 10 mechanically tested homografts after four different decontamination times. Elastic modulus, yield stress and energy at yield stress were calculated and compared between short (two to four days) and prolonged decontamination time (seven to nine, 28-30 or 60-62 days). Differences were analyzed with the Wilcoxon signed rank test. Study V included 32 homografts with biopsies collected at preparation (day 0) and after one, two, three, four, seven, 14, 21, 28 and 60 days of decontamination. Biopsies were studied using light microscopy (LM) and transmission electron microscopy (TEM) to evaluate cells, elastin, and collagen. Differences were compared between day 0 and a prolonged time (1-60 days) and analyzed with a Wilcoxon signed rank test.

Results.

I. Aortic homografts, young donor age and an ischemic time of 1-24 hours (compared to 0 hours) were identified as risk factors for early reintervention. There was no difference when extending the ischemic time to 48 hours compared to 24 hours.
II. Structural impairment of the homograft was not a risk factor for early reintervention.
III. Microbiological contamination before antibiotic decontamination during processing was not a risk factor for early reintervention or development of endocarditis.
IV. Elastic modulus and yield stress were significantly higher at two to four days compared to prolonged time. There were no significant differences in energy at yield stress at different times.
V. LM found a decrease in cell count after 60 days of decontamination. TEM found signs of cell degeneration after three days and beyond, signs of elastin degeneration after 21 days and signs of collagen degeneration after 28 days of decontamination.

Conclusions.

I. There is no harm in choosing a homograft from an older donor. It is possible to prolong the ischemic time to 48 hours without affecting the long-term outcome of homografts.
II. Current guidelines on structural impairments of homografts are acceptable.
III. There is no harm in using homografts that were contaminated before decontamination if follow-up cultures are negative.
IV. There could be some deterioration of the homograft resulting in decreased values of elastic modulus and yield stress with a prolonged decontamination time.
V. The earliest signs of elastin and collagen degeneration could be found after 21 and 28 days of antibiotic decontamination. Decontamination in a cold environment could be prolonged to at least 14 days without affecting homograft quality.

List of Publications

Paper I

Axelsson I., Malm T. Long-term outcome of homograft implants related to donor and tissue characteristics. The Annals of Thoracic Surgery. 2022 Jul 1; 106(1):165-71.

Paper II

Axelsson I, Malm T., Nilsson J. Impact of valve fenestration and structural changes in homografts on the long-term outcome in the recipient. Cell and Tissue Banking. 2021;22(3): p 399-408.

Paper III

Axelsson I, Malm T, Nilsson J. Does microbiological contamination of homografts prior to decontamination affect the outcome after right ventricular outflow tract reconstruction? Interactive CardioVascular and Thoracic Surgery. 2021;33(4): p. 605-613.

Paper IV

Axelsson I, Gustafsson A, Isaksson H, Nilsson J, Malm T. Impact of storage time prior to cryopreservation on mechanical properties of aortic homografts. Cell and Tissue Banking. 2023; doi.org/10.1007/s10561-023-10079-z.

Paper V

von Konow I, Eliasson A, Nilsson J, Malm T. Impact of Prolonged Storage Time on Homograft Ultra-Structures – An Attempt to Find Optimal Guidelines for Homograft Processing. Manuscript, submitted to Cell and Tissue Banking.

Populärvetenskaplig sammanfattning

Medfödda hjärtfel drabbar 1% av alla nyfödda barn. Vid flera olika typer av diagnoser saknas en normal förbindelse mellan höger hjärthalva och lungorna, vilket gör att hjärtat utsätts för hög belastning samtidigt som syresättningen blir dålig. Utan behandling leder detta problem till försämrad kondition och i många fall en för tidig död. Hjärtfelet åtgärdas med en operation då man återställer blodflödet genom att operera in en förbindelse som består av ett rör med en klaff i, som efterliknar den normala lungartären som vanligtvis kopplar samman högerhjärtat med lungorna. Det finns olika typer av rör man kan använda men i dagsläget anser man att en mänsklig donerad hjärtklaff, ett så kallat homograft, är ett av de bästa alternativen. Detta beror på att homograften har låg risk för infektioner, inte kräver någon blodförtunnande behandling samt att de leder till att man efterliknar den normala anatomin vilket resulterar i ett välfungerande blodflöde ut i lungorna.

Omhändertagandet av homograft sker på s.k. vävnadsbanker, där en av de största vävnadsbankerna i Skandinavien finns i Lund. Dessa vävnadsbanker tillvaratar hjärtan från avlidna personer och klipper ut hjärtklaffarna, behandlar dem med antibiotika och fryser sedan ner dem till -196°C då de kan förvaras i upp till 10 år. Från ett hjärta kan man utvinna två homograft, ett från högra och ett från vänstra sidan av hjärtat.

Det finns två stora nackdelar med homograft. Det första är att de har en begränsad hållbarhet efter operationen. Efter 20 år har knappt hälften av homograften utvecklat förträngningar eller läckage som kräver att man byter ut det på nytt. Hur länge ett homograft håller är väldigt individuellt, ibland kan det hålla längre är 20 år men hos många håller det betydligt kortare. Hos unga individer är hållbarheten sämre och hos individer under ett års ålder behöver homograftet ofta bytas ut inom bara ett eller ett par års tid. Den andra nackdelen är en begränsad tillgång på homograft. Det finns inte ett oändligt antal donatorer och strikta riktlinjer gör att många homograft inte uppfyller kriterierna för att kunna användas.

Syftet med denna avhandling är att närmare undersöka homograften och studera vilka faktorer som kan leda till sämre resultat hos patienterna samt om våra protokoll för omhändertagande av homograft är adekvat utformade. Tidigare forskning på detta område är begränsad, och många riktlinjer är i huvudsak baserade på erfarenhet och tyckande snarare än vetenskap. Vår hypotes är därför att många riktlinjer skulle kunna göras om och att vi idag kasserar onödigt många homograft för att vara på den säkra sidan.

Avhandlingen innehåller fem delarbeten. I delarbete nummer 1-3 har man studerat journaler från både homograft och patienter och analyserat vilka faktorer som skulle kunna ge bättre eller sämre resultat efter att man opererat in ett homograft. Det första delarbetet fokuserade på olika egenskaper hos individerna som donerar homograft samt olika faktorer i omhändertagandet. Detta arbete kunde

visa att ung ålder hos donatorn av ett homograft var en riskfaktor för sämre resultat och att homograft som hade donerats från äldre individer (>30 år) ofta höll längre efter det blev inopererat. När man undersökte olika faktorer i omhändertagandet studerade man bland annat den maximala tiden mellan det att donatorn avlider (och blodcirkulationen upphör) tills det att man tagit ut hjärtat från kroppen. Många vävnadsbanker har valt att sätta denna tid till maximalt 24 timmar, då man tänker sig att längre tid skulle kunna leda till att homograftet försämras. I Lund har man däremot valt att sätta den maximala tiden till 48 timmar vilket leder till att man kan ta vara på fler donatorer. Efter att en person avlider är det en hel del arbete som måste hinnas med för att kunna avgöra om hjärtat kan doneras. Man måste titta efter tidigare sjukdomar, ge anhöriga information och tid på sig att fundera på beslutet om donation, samt transportera kroppen till vävnadsbanken alternativt åka i väg för att ta ut hjärtat på plats där donatorn finns. Det är ofta svårt att hinna med allt detta inom 24 timmar och i Lund kom 41% av de använda homograften från donatorer som hade >24 timmar mellan dödstillfället och uttag av hjärtat. När man analyserade detta närmare kunde man se att dessa homograft hade lika bra resultat som de homograft med kortare tider. Det finns tidigare studier som har visat samma resultat, vilket talar starkt för att homograften inte försämras när man förlänger den maximala tiden till 48 timmar.

Det andra delarbetet fokuserade på små skador i homograftet. När man klipper ut homograften från ett hjärta hittar man ibland fettinlagringar eller förkalkningar i kärlet samt små hål i själva klaffbladen på hjärtklaffen. Enligt aktuella riktlinjer kan man acceptera små områden med fettinlagringar samt små hål i klaffen så länge man kan se att klaffen inte läcker när man testar den under prepareringen. Detta arbete kunde visa att denna typ av skador är en av de absolut vanligaste orsakerna till att man måste kassera homograftet. Man kunde också visa att de homograft som faktiskt accepterades trots små skador höll lika länge som de homograft som var helt fläckfria innan de blev inopererade. Detta talar för att vi med gott samvete kan fortsätta använda de riktlinjer vi har, där vi accepterar små skador.

Det tredje delarbetet fokuserade på kontamination av bakterier och svamp under omhändertagande av homograft. Odlingar för bakterier och svamp tas i samband med att man klipper ut homograften från hjärtat. Man lägger sedan homograften i en antibiotikalösning i minst 24 timmar och därefter tas nya odlingar innan man fryser ner dem. Många vävnadsbanker har en lång lista på bakterier och svampar som anses riskfyllda, och kasserar homograft som är kontaminerade med dessa arter även om de enbart förekommer innan antibiotikabehandlingen. I Lund accepteras de flesta bakterier och svampar som dyker upp innan antibiotikabehandling, förutom några sorter som är resistenta mot antibiotika och anses vara extra riskfyllda. I Lund var en tredjedel homograften kontaminerade innan antibiotikabehandling, men 98% var fria från bakterier och svamp efter antibiotikabehandlingen. Alla homograft som har positiva odlingar efter antibiotikabehandlingen kasseras, både i Lund och på andra vävnadsbanker. Studie nummer tre kunde visa att homograft som varit kontaminerade innan antibiotikabehandling hade lika bra resultat som de homograft

som inte hade varit kontaminerade. Man kunde också se att kontamination innan antibiotikabehandling inte ledde till någon ökad infektionsrisk hos patienterna, vilket talar för att den antibiotikabehandling som används är effektiv.

De fjärde och femte delarbetena fokuserar på hur länge homograftet bibehåller sin kvalité och hållfasthet under tillvaratagningsprocessen och hur strikta tidsgränser man behöver använda. Det fjärde delarbetet undersökte hållfastheten i homograft efter olika lång tid i kylskåp innan nedfrysning. Man använde homograft som tillvaratagits på normalt sätt och klippte sedan bitar från homograftet efter 2–4 dagar (vilket motsvarar den normala tiden i kylskåp innan infrysning) samt efter 7–9 dagar, 28–30 dagar och 60–62 dagar. Därefter testade man hållfastheten genom att sätta bitarna i en maskin som drog i dem tills de gick sönder. Med hjälp av ett datorprogram kunde man beräkna elasticiteten i vävnaden samt hur stark kraft man kunde dra med innan de gick sönder. Resultaten visade att vävnaden var som mest motståndskraftig efter 2–4 dagar, och att resultaten försämrades vid 7–9 dagar och längre. Detta resultat talar för att homograftets strukturella byggstenar försvagas vid förlängd tid i kylskåp innan nedfrysning.

Under det femte delarbetet undersökte man 32 homograft i mikroskop, både i ljusmikroskop och elektronmikroskop, där man kunde få skarpa bilder med upp till 87000x förstoring. Man använde homograft som tillvaratagits på normals sätt, och klippte ut vävnadsbitar i samband med prepareringen (som kallas för dag 0) samt efter 1, 2, 3, 4, 7, 14, 21, 28 och 60 dagar i kylskåp. Man analyserade sedan celler och olika typer av fibrer som är viktiga för homograftets elasticitet och hållfasthet (elastin och kollagen). Analysen visade att cellerna började brytas ner redan efter 3 dagar, men att elastin såg normalt ut i upp till 21 dagar och kollagen såg normalt ut i upp till 28 dagar. Det finns flera studier som talar för att cellerna inte verkar ha stor betydelse för homograftets hållbarhet, men att elastin och kollagen kan vara viktiga för normal funktion. Resultatet visar tydligt att dessa fibrer är helt intakta i upp till 21 dagar i kylskåp, vilket både underlättar arbetet och sänker arbetskostnaden för vävnadsbanker. När man kan förlänga tiden i kylskåp behöver man inte bedriva arbete på kvällar och helger för att hinna frysa in homograften i tid, utan kan fokusera arbetet till normal arbetstid.

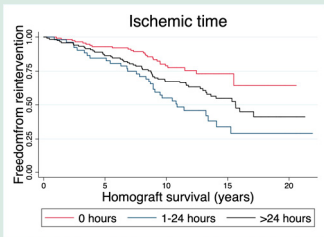
Sammantaget så verkar homograftet hålla god vävnadskvalité hos både äldre donatorer och vid förlängd omhändertagningstid, vilket både ökar antalet donatorer och underlättar arbetet på vävnadsbankerna. Man kan acceptera små skador i homograftet, förutsatt att hjärtklaffen uppvisar god funktion och inte läcker. Man behöver inte kassera homograft som uppvisar växt av bakterier eller svamp innan antibiotikabehandling, förutsatt att uppföljande kontroller är negativa.

Thesis at a glance

	Research aim	Methods	Key Results
I	To evaluate different donor characteristics and homograft processing methods in relation to the long-term outcome in recipients of homografts.	Retrospective correlational study. Analysis with Kaplan-Meier method with a log-rank test and Cox proportional hazard regression.	A homograft ischemic time of >24 hours did not have a higher reintervention rate compared to a shorter ischemic time (HR 1.1, 95% CI 0.67-1.8). Young donor age is an independent risk factor for early reintervention (HR 0.97, 95% CI 0.95-0.98)
II	To evaluate the prevalence of small structural defects, such as small cusp fenestrations and atheromatosis of the vessel wall in homografts, and their impact on the long-term outcome in recipients of homografts.	Retrospective correlational study. Analysis with Cox proportional hazard regression.	Minor structural changes, such as small fenestration or minor atheromatosis, were not associated with a higher reintervention rate in recipients compared to homografts without remarks.
III	To evaluate the prevalence of microbiological and fungal contamination of homografts during procurement and their impact on the long-term outcome and risk of endocarditis in recipients of homografts.	Retrospective correlational study. Analysis with Cox proportional hazard regression.	Contamination before antibiotic decontamination was not associated with a higher reintervention rate nor a higher rate of endocarditis compared to homografts that were never contaminated.
IV	To evaluate the mechanical properties of homografts after different decontamination intervals, to determine if a prolonged time of antibiotic decontamination had any effect on the mechanical properties of the homograft.	Prospective experimental study, investigating the effect of a prolonged decontamination time on the mechanical properties of the homograft.	Elastic modulus decreased significantly when prolonging antibiotic decontamination to >7 days before cryopreservation. Yield stress and Energy at Yield stress varied at different times.
V	To evaluate homograft structures and ultrastructures after different decontamination intervals, to determine if it is possible to find a maximum time of antibiotic decontamination where the homograft keeps its structural integrity and quality.	Prospective experimental study, investigating the effect of a prolonged decontamination time on cells, elastic fibers and collagen in the homograft.	Light microscopy only identified a decreased cell count after 60 days of decontamination. Transmission electron microscopy identified cell deterioration after 3-4 days, elastic fiber deterioration after 21 days and collagen deterioration after 28 days of decontamination.

Conclusion and significance

I



Cold ischemic time before heart collection can be expanded to 48 hours without affecting the long-term outcome in recipients. There is no harm in choosing a homograft from an older donor. Allowing for prolonged ischemic time expands the possible donor pool.

II

Structural changes	Multivariable analysis	
	HR	95% CI
No changes	1.0	
Fenestrations	0.46	0.11-1.9
Atheromatosis	0.80	0.25-2.6

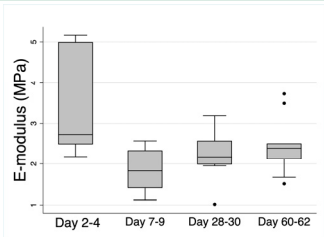
Current guidelines on structural impairment seem acceptable considering long-term outcome.

III

Microbiological contamination	Multivariable analysis	
	HR	95% CI
No	1.1	0.73-1.7
Low-risk	1.0	
High-risk	1.6	0.87-2.8

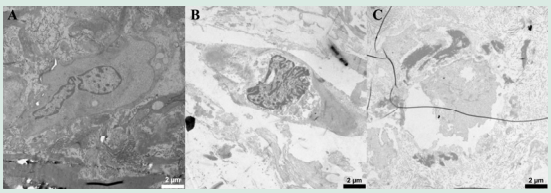
Contamination of homografts before decontamination does not affect long-term results. Homografts that are contaminated before decontamination do not need to be discarded if follow-up cultures are negative, which increases the number of homografts available for transplantation.

IV



Elastic modulus decreases with a prolonged ischemic time, indicating decreased stiffness of the tissue. The clinical significance of this result remains to be clarified.

V



Transmission electron microscopy identified early cell degeneration. Elastic fibers and collagen were more resistant to degeneration, and showed no deterioration until 21 and 28 days of decontamination. Decontamination time could be extended to at least 14 days without affecting the quality of the tissue matrix.

Introduction

Homografts

One in a hundred children is born with a congenital heart defect, with a wide range of diagnoses and severity. Pediatric cardiac surgery has developed rapidly since the 1950s when the first heart-lung machine was launched, and the prevalence of children surviving into adulthood is steadily growing. In 2017, 97% of children with congenital heart disease survived to adulthood if they had access to pediatric cardiology care and cardiac surgery (1–3). Improved survival results in a growing population of adults with congenital heart disease (ACHD) who require adequate follow-up and occasional surgical reinterventions to treat their heart defect (4,5).

Several congenital heart defects involve stenosis or insufficiency in the right ventricular outflow tract (RVOT), requiring surgical repair. These heart defects require both primary corrections and reinterventions during a lifetime. In ACHD, pulmonary valve (PV) replacement and RVOT reconstruction are two of the most common reintervention procedures (6). Reconstruction often requires a conduit between the pulmonary artery and the right ventricle (RV) and different options are available. One of the preferred options during RVOT reconstruction is the use of a human-donated heart valve, the homograft (5,7).

In its original meaning, the term ‘homograft’ (or its synonym ‘allograft’) refers to an organ or tissue that is donated from one individual where the receiver is of the same species. However, in medical literature, ‘homograft’ most often refers to a transplanted human PV or aortic valve (AV). Therefore, when the term ‘homograft’ is used further on, it will refer to the human heart valve graft (Figure 1).

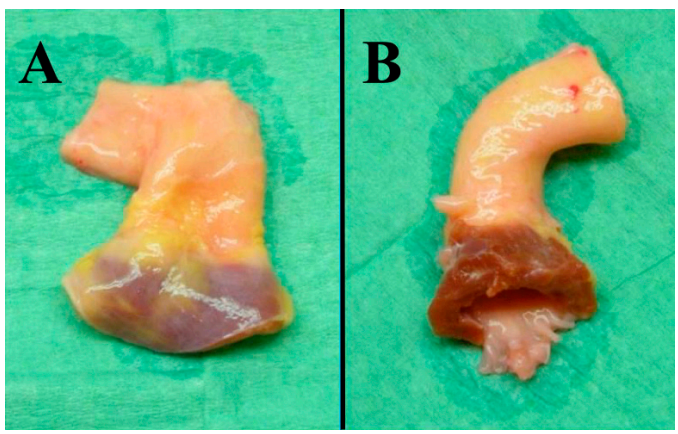


Figure 1.
A: Pulmonary homograft. B: Aortic homograft.

Homografts were first used in cardiac surgery in 1956 when Dr. Gordon Murray used an aortic homograft in the descending aorta to treat a patient with aortic regurgitation (8). Ten years later, Dr. Donald Ross used a homograft in the RVOT for the first time, treating a young boy with pulmonary atresia (PA) (9). Ever since, the use of homografts has expanded, with a wide variety of indications, such as:

- Primary corrections of congenital defects such as PA, transposition of the great arteries, truncus arteriosus, absent pulmonary valve syndrome, and isolated RVOT obstructions or insufficiencies (5,10).
- Reoperation of patients with primary corrected Teratology of Fallot and development of pulmonary stenosis (PS) or pulmonary insufficiency (PI) (11,12).
- Reoperation of an earlier implanted conduit that has developed stenosis or insufficiency (10,13).
- Reconstruction of the left ventricular outflow tract (LVOT) in patients with extensive AV endocarditis (14).
- Reconstruction of the RVOT after using the pulmonary artery as an autograft in the aortic position during the Ross procedure in patients with LVOT disease (15).

Long-term results

Patients receiving a homograft in the RVOT have shown excellent long-term survival (16–18). The main advantages of the homograft are good hemodynamic performance, low risk of endocarditis compared to alternative conduits, and no need for anticoagulative therapy which is especially beneficial in active children and women who are planning to become pregnant during their lifetime (15,19,20). Homografts perform well during pregnancy and without risk of accelerated degeneration (6). However, there are two disadvantages of the homograft. The first is the limited durability, with the development of stenosis and/or insufficiency over time. Long-term follow-up shows 1-, 5-, 10-, and 20-year freedom from reintervention of 95-99%, 76-94%, 58-86% and 32-56% respectively (4,13,17,18,21–27). The results from different studies show a high variation in outcome, partly depending on the heterogenicity of patient groups with different patient ages, levels of diagnostic severity and types of procedure. For example, studies including patients who underwent the Ross procedure tend to have better outcomes, since freedom from reintervention in middle-aged patients undergoing the Ross procedure could be as high as 92% after 16 years (28). When investigating the other end of the spectrum, studies looking at young patients (<1 year) with complex diagnoses who receive a homograft in the RVOT showed 1-, 5-, and 10-year freedom from reintervention of 75-88%, 25-45% and 38% (29–31).

Quality of life in patients undergoing RVOT reconstruction is comparable to the general population. Patients score slightly lower on physical functioning and general

health, but show similar scores for mental health and emotional and social function (25).

The second disadvantage of the homograft is limited availability. All sizes are not always available, and the surgeon could be forced to use a different conduit or valve size due to a lack of homografts (32,33).

Risk factors for early reintervention

Aortic or pulmonary homografts

Repeated studies show that aortic homografts need earlier reintervention compared to pulmonary homografts, usually because of accelerated calcification and development of valve stenosis (13,18,23–25,30,34–36).

Recipient age, donor age and homograft size

Younger recipients, younger donors, and smaller homograft sizes are well-defined risk factors for earlier reintervention (4,16–18,20,21,23–27,29–31,35–39). Since all three variables highly correlate with one another in all materials, it is difficult to assess if one variable contributes more than the others to the increased risk. Younger patients tend to develop calcification and stenosis faster (40). The reason for this is unknown, but some studies suggest a stronger immune response against the homograft in small children (40,41). Outgrowth of the homograft occurs but is much more uncommon than early development of conduit stenosis and is not the reason for the poor performance of small homografts (42). Hence, over-sizing the homograft will not improve performance in young recipients but will rather increase the risk for early degeneration (36,38).

Even if young age is associated with early reintervention, delay of surgery is associated with increased mortality. If the heart defect is not corrected, adverse remodeling of the RV could lead to irreversible heart failure over time (4).

Conclusively, RVOT dysfunction should be addressed early and optimal timing for surgery must be individualized.

Complexity of diagnosis

Diagnostic complexity is associated with earlier reinterventions, especially if the homograft must be implanted in an extra-anatomic position (4,13,23,26,26,31). For example, truncus arteriosus is a heart defect with high anatomical complexity with a higher risk of early reintervention after correction (39,43). The other end of the spectrum is patients undergoing the Ross procedure, a subgroup that shows one of the longest homograft durations. During the Ross procedure, the homograft is implanted in a healthy RVOT with normal anatomical conditions (23,29).

AB0-mismatch

Studies investigating the importance of blood group matching between donor and recipient have shown contradictory results. Most studies have not been able to show any correlation between blood group mismatch and earlier reintervention (17,30,34,44), and there is no correlation between the infiltration of immune cells in explanted homografts and blood group mismatch (45). Still, some studies did see a correlation between mismatch and earlier reintervention, and results remain contradictory up to this day (37,46). Currently, there is not enough evidence to argue that AB0-matching needs to be considered when choosing a suitable homograft.

Other conduits used in right ventricular outflow tract reconstruction

Due to the known disadvantages of homografts, several attempts have been made to produce a better, off-the-shelf alternative to use in RVOT reconstruction and PV replacement.

Xenografts

There are several different xenograft conduits available, but the Contegra® (Medtronic Inc., Minneapolis, MN, USA) is the one that has gained most popularity. The Contegra conduit is a bovine jugular vein conduit with a tricuspid valve that is available in sizes 12-22 mm. Its main advantage compared to homografts is the off-the-shelf availability (47). The results after Contegra implantation have varied. Some centers have experienced inferior results after Contegra implantation compared to homografts, usually because of the development of stenosis in the distal anastomosis of the conduit (26,48,49). Other centers have found comparable results after homograft and Contegra implantation (31,50–52). Inferior performance of the Contegra is more evident when comparing them to pulmonary homografts only, instead of comparing them to homografts in general (53). One clear disadvantage of the Contegra conduit is a higher incidence of infective endocarditis (IE) compared to homografts. The cumulative incidence of IE after implantation was 6.0-13% for Contegra compared to 0.80-1.3% for homografts after 5-7.5 years of follow-up (27,43,54–56).

Transcatheter pulmonary valves

Transcatheter pulmonary valve implantation (TPVI) is used for RVOT dysfunction, mainly as a reintervention for previously implanted conduits but also as a primary correction when possible. The first approved device was the Melody™ valve (Medtronic plc, Ireland) which was introduced in 2006. Follow-up after implantation shows a low incidence of mortality, comparable to surgical RVOT reconstruction (57). Freedom from reintervention was 87% at four years and 79% at six years which is comparable to the homograft outcome (58). The main disadvantage of the Melody valve has been the high prevalence of IE, where the

cumulative incidence of IE was 11-15% after five years of follow-up, comparable to Contegra conduits but much higher than in homografts (54,55,58).

Pericardial tissue valves

Several different pericardial tissue valves that were originally developed for AV replacement have been tried in the pulmonary position. These valves are often referred to as "biological heart valves", where the leaflets are made from bovine or porcine pericardium. Like the Contegra valve, short- and midterm results are comparable to homografts when including all homografts, but freedom from reintervention is inferior compared to pulmonary homografts (59). In recent years, the Inspiris Resilia bioprosthesis (Edward Lifesciences LLC) has gained popularity due to its results with low gradients in the aortic position. One center has shown excellent results with the Inspiris valve in the pulmonary position with no structural valve degeneration after 2.5 years follow-up (60). Other centers have not been able to replicate this result, but rather show that the Inspiris valve has a significantly higher incidence of early moderate to severe pulmonary regurgitation compared to other conduits (61–63).

Mechanical valves

Mechanical valves have been rarely used in the pulmonary position due to the elevated risk of thrombosis in a low-flow system, but recent studies suggest that the risk of thrombosis is low if adequate anticoagulation is used (64,65). Long-term risk for reintervention seems to be similar or superior compared to biological valves (homografts, xenografts or pericardial tissue valves), but studies are small with heterogeneous groups (64,65). Today, there are no recommendations on using a mechanical valve in the pulmonary position (5).

Decellularized homografts

To increase the durability of homografts, decellularized homografts (DHs) were introduced at the beginning of the 21st century, gaining popularity and interest. The rationale behind using DHs is that they do not elicit an immune reaction after implantation, thus prolonging the lifespan of the tissue (66,67). Studies of DHs have shown similar, low mortality rates and promising reintervention rates compared to other types of conduits (68).

It has been clearly shown that the immune response is significantly weaker or completely absent after implantation of a DH compared to a regular homograft. The expression of human leukocyte antigen (HLA) antibodies post-implantation is lower, reflecting the lack of immunogenicity in DHs (69,70). Except for the potential benefit of prolonged longevity of DHs due to a lower immune response, this could also benefit the subset of patients who will eventually require a heart transplant, since the levels of HLA antibodies are significantly lower up to at least one year after DH implantation (69,71).

Many studies claim better outcomes of DHs compared to regular homografts but looking closer at the data, most studies does not investigate comparable groups. Boethig et al. showed significantly better freedom from explantation and degeneration for DHs, but the DHs group were operated in a more modern time period compared to the group with regular homografts (2014-2016 vs. 1988-2005), DHs consist of pulmonary homografts only while it was not stated how many of the regular homografts that are aortic vs. pulmonary, and even if the compared groups were of the same sample size at the beginning of follow-up (235 patients each), only four of the DHs patients were followed for a total of 10 years compared to 62 of the regular homografts (72). Bibevski et al. showed more comparable groups with superior long-term outcome of the DHs, especially among younger patients. In their study, the DHs had only a slightly larger diameter and older recipients compared to regular homografts, but information about the proportion of pulmonary vs. aortic homografts in the regular homograft group was missing, while the DHs were pulmonary homografts only (66).

Other studies show comparable reintervention rates of DHs and regular homografts but demonstrate a lower degree of stenosis and insufficiency development in DHs. Ruzmentov et al. showed better performance of DHs compared to regular pulmonary homografts after five and 10 years according to homografts dysfunction (peak gradient >40 mmHg or insufficiency grade 3-4, representing flow reversal in the conduit and the pulmonary arteries respectively) and reintervention in two comparable groups (73). Etnel et al. compared the use of pulmonary homografts and pulmonary DHs in the Ross procedure in propensity-score matched groups. The outcome after eight years showed no differences between the groups according to reintervention, but DHs had a significantly slower progression of stenosis compared to regular pulmonary homografts (74). Tavakkol et al. investigated pulmonary homografts compared to pulmonary DHs in propensity-score matched groups with a young mean age (5 years). At 19 months of follow-up, reintervention rates were low and similar in both groups, but the mean peak gradient and grade of insufficiency were significantly lower in the DHs group (75). Brown et al. investigated pulmonary homografts compared to pulmonary DHs in both Ross and other RVOT implantations with four years follow-up and showed a lower degree of peak gradient (19 vs. 22 mmHg) in the Ross group and a lower degree of PI in both groups of DHs compared to regular pulmonary homografts (76).

Finally, some studies have not been able to find any differences in outcome after implantation of DHs compared to homografts, but they all show trends (not significant) for better performance of DHs (71,77,78). No one has demonstrated an inferior outcome of DHs compared to regular homografts or any other conduit.

In conclusion, decellularized pulmonary homografts show promising results, but longer follow-up and better study designs are required before making any major conclusions about their hypothesized superiority.

Tissue banks

The availability of homografts is dependent on tissue banks that procure, prepare, control, cryopreserve, and distribute homografts for clinical use. In Europe, tissue banks develops their protocols from guidelines developed by the European directorate for the quality of medicine and health care (EDQM) (19,79). As described below, some guidelines have been developed from rigid scientific evaluation, but many have been developed from experience and expert opinion only. This results in a high influence of local routines when developing protocols. The discard rate ranges from 19 to 65% of all homografts in different tissue banks, reflecting differences in protocols. The most common reasons for discard in most tissue banks are microbiological contamination, structural impairment of the tissue, or medical contraindications (79). Due to the limited availability of homografts, it is of great importance to maximize the use of available tissue without compromising its quality.

Processing methods

As mentioned above, homograft processing differs between tissue banks, but the basic structure is the same and consists of the following steps (19):

1. Donor hearts are collected by surgeons or specially trained personnel under sterile conditions, either in an operating theater or a morgue.
2. The heart is transported to the tissue bank in a sterile and cold environment (2-8°C).
3. The homografts are prepared from the heart under sterile conditions at the tissue banks, often in a clean room with a laminar air flow cabinet. The homografts are macroscopically inspected for impairments (Figure 2). Samples are retrieved for microbiological and sometimes morphological analysis (depending on the tissue bank).
4. The heart is sterilized in an antibiotic solution where the antibiotic cocktail, time of sterilization and temperature differs between tissue banks (79). Additional cultures are retrieved after sterilization.
5. Homografts are cryopreserved to -196°C and are stored in the liquid nitrogen phase for a maximum of five to 10 years.

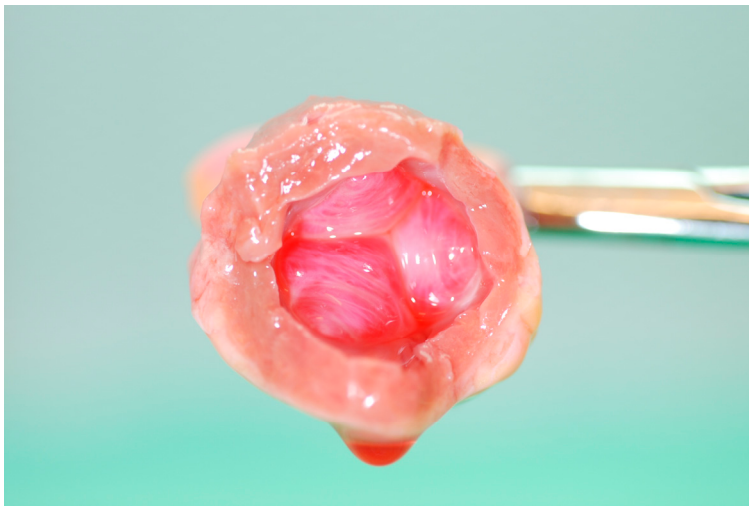


Figure 2. Inspection of valve cusps during homograft preparation.

Homograft donors

There are three main types of homograft donor:

- Non-heart-beating donors (NHBD). Homografts donated after circulatory death. These donors have an episode of warm and/or cold ischemic time before heart explantation, which is considered in the ischemic time section.
- Multi-organ donors (MOD). Homografts donated after brain death. The heart is used for homografts when it is unsuitable for complete heart transplantation. These hearts are circulated until explantation, meaning that the ischemic time for the heart does not start until the explantation.
- Domino donors. Homografts donated from the recipient of a heart transplantation, where the explanted heart can be used for homograft donation.

Total ischemic time

The EDQM has the following recommendations on time limits during homograft processing (19):

- The time from donor circulatory arrest until heart explantation should be a maximum of 24 hours, with a maximum of six hours of warm ischemic time.
- The time from heart explantation until dissection and decontamination should be a maximum of 24 hours.
- The total ischemic time (donor circulatory arrest until cryopreservation of homografts) should be a maximum of 72 hours.

In 1987, the cryopreservation technique was introduced, which led to a longer possible storage time and better outcomes for patients receiving a cryopreserved homograft compared to a fresh homograft stored at 4°C. These results started the debate that early cryopreservation could maintain cellular viability which could enhance the homograft performance and durability after implantation (80–82). An issue with these studies is that they do not define how long the fresh homografts were stored before implantation, but according to other studies the commonly accepted time frame seems to have been eight to 12 weeks at 4°C (45).

Decontamination routines and microbiological burden

The EDQM has no strict recommendation on what antibiotic regime to use; they only recommend that every tissue bank should develop routines to evaluate the homograft for microbiological burden both before and after antibiotic decontamination and develop an antibiotic cocktail suitable for the local environment. Most tissue banks decontaminate their homografts in a cold environment, but about a third of tissue banks in Europe decontaminate their homografts at room temperature or at 37°C (79).

Some contaminants can be accepted if found before decontamination, but all contaminants that are found post decontamination should lead to the discard of the current homograft with an additional risk assessment for all other tissues from the same donor. Table 1 demonstrates a list of microbes and fungi that, according to the EDQM, should always lead to the discard of cardiovascular tissue regardless of whether found before or after antibiotic decontamination (19).

Table 1. List of microbes and fungi that, according to the EDQM, should always result in the discard of cardiovascular tissue regardless of whether found before or after antibiotic decontamination (19).

Staphylococcus aureus and *lugdunensis*
Streptococcus spp. and *Enterococcus* spp.
Clostridium spp.
Enterobacteriaceae (eg. *Escherichia coli, Enterobacter* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp.
Yeast and filamentous fungi (molds).

Many tissue banks follow these recommendations, leading to microbiological contamination often being one of the main reasons for the discard of homografts (79,83).

The contamination rate before decontamination varies from 14 to 84% in different tissue banks. In general, contamination rates are higher in NHBD compared to MOD (84–90). The most common microbiological findings are low virulent skin flora, such as coagulase-negative *Staphylococcus* and *Cutibacterium acnes,* followed by more virulent bacteria such as *Streptococcus* spp. and *Clostridium* spp. (85–88,90).

At the tissue bank in Lund, four cultures are collected at three different times during processing. The first is a tissue culture that is retrieved after preparation but

prior to antibiotic decontamination. The homograft is then placed in the antibiotic cocktail at 24-96 hours at 4°C. After decontamination, the homograft is rinsed twice in Ringer-acetate to remove residual antibiotics and then put in a cryoprotectant solution with dimethylsulfoxid for 90 minutes. Prior to packing, additional cultures are retrieved from the tissue and from the cryoprotectant solution. At implantation, tissue cultures are retrieved from the homograft in the operating room.

The growth of multi-resistant bacteria (MRSA, VRE, ESBL) and pseudomonas leads to the discard of tissue at any point in the process. All other microbes are accepted if found before antibiotic decontamination, which differs from the EDQM guidelines. Positive cultures after antibiotic decontamination always result in the discard of tissue.

Macroscopic control

Minor structural impairment of the homografts can be accepted according to the EDQM. Such impairments include small fenestrations in the rim of the cusps if they do not affect the functional competence of the valve (Figure 3). Minor atheromatosis of the vessel wall can be accepted as well. These guidelines are developed from experience and expert opinion only (19). Tissue quality is assessed by macroscopic and microscopic inspection. Structural impairment is the main reason for the discard of homografts at many tissue banks, including the tissue bank in Lund (91).

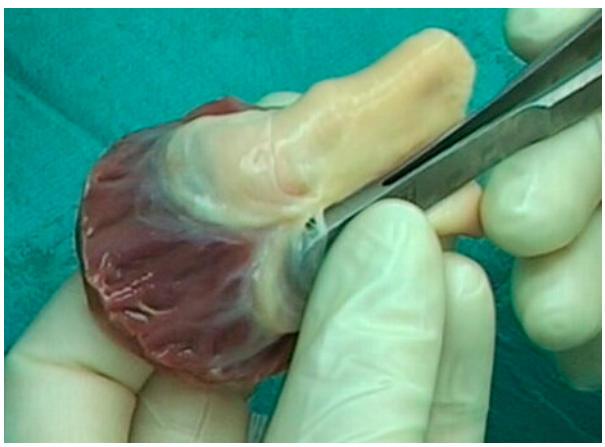


Figure 3. Examples of fenestrations that are accepted.

During preparation at the tissue bank in Lund, the cusps and vessels are closely reviewed for damage and insufficiency. The homograft is turned inside-out to inspect the cusps. Fenestrations are measured with a sterile ruler. Small (<3x2 mm) fenestrations are accepted if localized in the rim of the cusps, but homografts with larger fenestrations are discarded. If localized peripherally in the cusps, close to the commissures, more than one fenestration can be accepted but if localized more

centrally on the cusps free margin, fenestration in one cusp is only accepted to avoid insufficiency. The vessel wall is inspected for atheromatosis. Soft atheromatosis in smaller parts of the vessel wall is accepted. Homografts with ulcerated plaque, hard atheromatosis, atheromatosis that is loose from the vessel wall or widespread soft atheromatosis are discarded. After inspection of the vessel wall, the coaptation of the cusps is inspected. Minimal leakage is accepted if the cusps show proper coaptation. Signs of moderate to severe leakage, prolapse of the cusps, valve insufficiency or anatomical abnormality result in discard of the tissue.

Cryopreservation

For long-term storage, the homograft is immersed in a cryoprotective solution and then cryopreserved to -196°C and stored in the liquid phase of nitrogen. Cryopreservation preserves the cell viability and extracellular matrix but could lead to some deterioration of the collagen fibers (92). These changes do not seem to impact the mechanical properties of the tissue, suggesting that the strength and function of the fibers are still intact (93). The EDQM recommends a maximum of five years of storage after preservation, but studies have shown that homografts show normal tissue integrity and mechanical properties after up to 10 years of storage (93,94).

Routines at the tissue bank in Lund

As mentioned, different tissue banks tends to deviate from the European guidelines depending on local experience and opinion. When developing local protocols at the tissue bank in Lund, guidelines were taken into consideration, but local adaptions have been made. Local adaptions are made according to personal experience, in cases where the guidelines do not have any scientific evaluation of their current recommendation. A summary of local protocols and guidelines is shown in Table 2. The tissue bank in Lund uses NHBD, MOD and domino donors. All homografts are dissected, inspected, and measured in a laminar airflow cabinet.

Table 2. Overview of processing routines at the tissue bank in Lund and comparison to European guidelines (19).

Guideline	Tissue bank Lund	European guidelines
Total ischemic time	216 hours	72 hours
Prior to explantation	48 hours (maximum 6 hours warm)	24 hours (maximum 6 hours warm)
Explantation to preparation	72 hours	24 hours
Decontamination time	24-96 hours	6-48 hours
Cryopreservation time	10 years	5 years
Macroscopic control (impairments accepted)	Small peripheral fenestrations (<3x2 mm). Small central fenestrations (<3x2) in one cusp only. Functional competence must be ensured. Soft atheromatosis in small areas.	Small fenestrations (size not defined), if functional competence is ensured. Minimal presence of calcification.
Antibiotic cocktail	Medium 199 Earle's salt (200 ml) Vancomycin (100 mg) Gentamicin (106 mg) Amphotericin B (50 mg)	Any cocktail that is validated to be effective on the local bioburden
Microbes leading to discard	MRSA, ESBL, VRE and pseudomonas at any stage. All positive cultures after antibiotic decontamination.	All microbes from Table 1 at any stage. All positive cultures after antibiotic decontamination.

Homografts in the microscope

Normal tissue architecture in the aortic wall is described as the presence of three aortic layers, the intima, media, and adventitia. The media consists of lamellar units with smooth muscle cells, collagen, and proteoglycans between long, intact elastic fibers (95). Pulmonary arteries have a very similar structure, but the elastic fibers are sparse, short, and irregular, resulting in a less organized architecture with fewer lamellar units (Figure 4) (96,97). Healthy vessels have intact, long elastic fibers with surrounding fibrillin to anchor mature elastic fibers to surrounding tissue. Collagen is arranged in dense bundles with a banding pattern in the fibers, called D-bands, that can be identified at high magnification in electron microscopy (95,98–100).

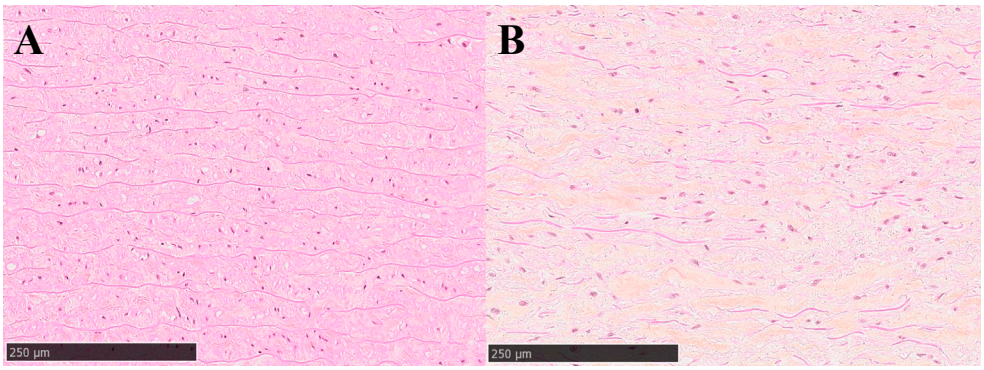


Figure 4. A: Normal media of the aortic wall. Lamellar units are seen with long, intact elastin fibers. B: Normal media of the pulmonary artery wall. Elastic fibers are more sparse, short, and irregular. Erythrosine saffron stain. 10x magnification.

Mechanical properties of the homograft

Elastin and collagen fibers provide strength and elasticity to the homograft vessel wall and cusps. Elastin is elastic fibers that enable deformation and extension of the tissue during systole and return to its original configuration at rest. Collagen fibers are crimpled at rest, enabling elongation at minimal stress but providing strength to the tissue at increased workload. Aortic homografts have higher vessel wall strength compared to pulmonary homografts (94,99), but the pulmonary artery is more elastic than the aorta (97).

Autolytic changes

As discussed above, increased total ischemic time leads to degenerative processes in the homograft that could lead to disadvantages after implantation.

When cells are exposed to starvation and ischemia, they start to degenerate. Chromatin starts to condense within the cell nuclei. Simultaneously, cell content

starts to vacuolize, and cells start to shrink and detach from surrounding tissue (101–103).

Elastic fiber degeneration is shown as increased complexity, splitting, fragmentation, and thinning of the fibers (95,104).

Collagen fiber degeneration consists of increased fiber waviness, disrupted fiber orientation, and disrupted D-bands with reduced contrast (105).

Summary of the thesis aims

While some parts of the homograft processing are well studied, some processes have insufficient or a complete lack of scientific evaluation. Today, many guidelines on homograft processing are based on experience and expert opinion only. This thesis aimed to evaluate donor and homograft processing variables and their impact on homograft longevity and quality. The overall objective was to optimize homograft processing to maximize the use of available homografts without negatively impacting quality and safety.

Study I	To evaluate different donor characteristics and homograft processing methods in relation to the long-term outcome in recipients of homografts.
Study II	To evaluate the prevalence of small structural defects, such as small cusp fenestrations and atheromatosis of the vessel wall in homografts, and their impact on the long-term outcome in recipients of homografts.
Study III	To evaluate the prevalence of microbiological and fungal contamination of homografts during procurement and their impact on the long-term outcome and risk of endocarditis in recipients of homografts.
Study IV	To evaluate the mechanical properties of homografts after different decontamination intervals, to determine if a prolonged time of antibiotic decontamination had any effect on the mechanical properties of the homograft.
Study V	To evaluate homograft structures and ultrastructures after different decontamination intervals, to determine if it is possible to find a maximum time of antibiotic decontamination where the homograft retains its structural integrity and quality.

Methods

Ethical approval

The Regional Ethical Review Board in Lund, Sweden approved all studies. The identification number of the application was 2017/133 with a complementary application for studies IV and V with identification number 2018/568.

Donor and homografts

All homografts used for implantation in studies I-III and analysis in studies IV-V were distributed from the tissue bank in Lund. In study I, homografts implanted in the RVOT at Skane University Hospital during 1995-2008 were included. In studies II and III, the time interval was expanded to 1995-2018. Data on homografts were collected from local registers. All data in studies I-III was collected retrospectively.

For studies IV and V, prospective collection of homografts was conducted. Homografts unsuitable for transplantation due to structural impairment were included in the studies if the donor accepted the donation for scientific purposes. The procurement, preparation, decontamination, and cryopreservation were carried out according to standard protocols unless stated otherwise.

Homograft preparation for study IV

Ten aortic homografts were collected during 2020-2021. The inclusion criteria were aortic homografts and all types of donors between 18 and 65 years of age. Aortic homografts were chosen due to better availability (more aortic homografts are discarded due to structural impairment compared to pulmonary homografts). At preparation, the aortic vessel wall was measured with a ruler and then cut longitudinally with surgical scissors into a total of 12 samples from each homograft (Figure 5).

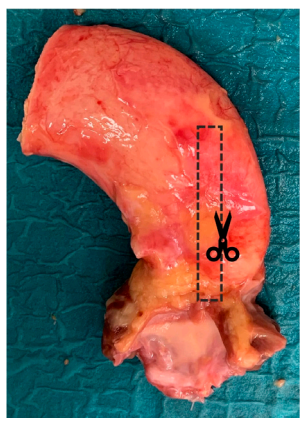


Figure 5. Collection of aortic vessel wall samples in study IV.

Three of the samples were cryopreserved after two to four days of antibiotic decontamination. These samples were used as reference samples since they were prepared, decontaminated, and cryopreserved according to the local standard protocol for homograft processing. The remaining nine samples were left in the antibiotic solution and cryopreserved in groups of three samples after seven to nine days, 28-30 days and 60-62 days. The seven to nine-day group was chosen to see if a small difference in time compared to the reference group would affect the result. The group of 60-62 days was chosen since this was the maximum time used when homografts were stored fresh prior to the introduction of cryopreservation in 1987 (81). The group of 28-30 days was chosen since it was in between the first and last groups.

Conclusively, each homograft generated 12 samples, where three samples were cryopreserved at each time investigated. Each time group had a total of 30 samples, three replicates from each of the 10 homografts (Figure 6).

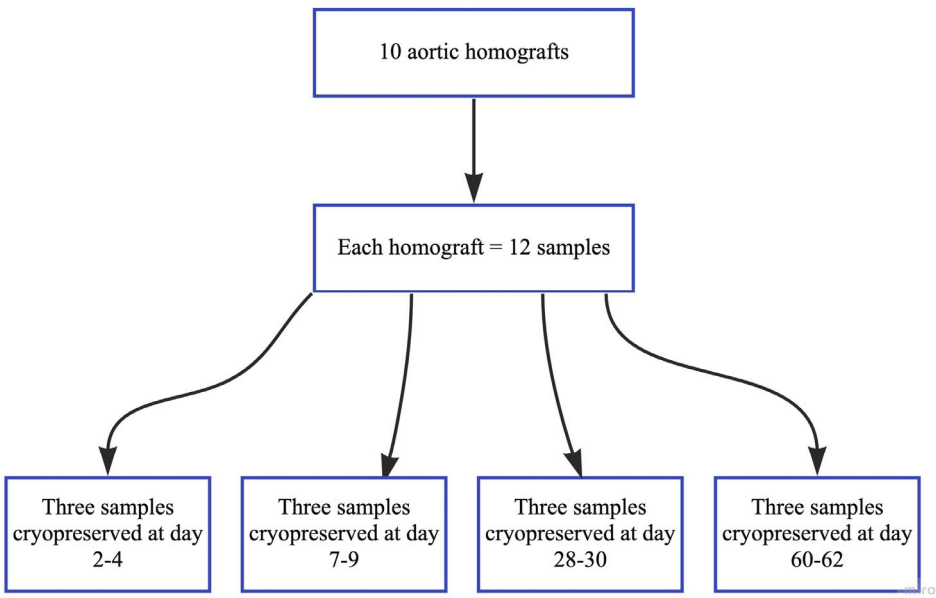


Figure 6. Flow chart of homograft sample collection for study IV.

Homograft preparation for study V

Twenty aortic and 12 pulmonary homografts were collected during 2019-2022. Inclusion criteria were MOD and age 18-65 years. MOD were chosen to avoid an impact from different ischemic times before heart explantation, which is the case in NHBD.

The first biopsies from the homograft vessel wall were retrieved in conjunction with the preparation procedure, prior to antibiotic decontamination. Three biopsies, 0.5x0.5 cm, were collected for light microscopy (LM) and one biopsy, 0.1x0.5 cm, was collected for transmission electron microscopy (TEM). The first biopsies were defined as "Day 0". The homograft was then put in a regular antibiotic solution and stored at 4°C according to standard protocols. Additional biopsies were retrieved after one, two, three, four, seven, 14, 21, 28 and 60 days in the antibiotic solution.

Homograft recipients

All recipients of a homograft in the RVOT that was implanted at the Pediatric Cardiac Surgery Unit or the Department of Cardiothoracic Surgery at Skane University Hospital during 1995-2008 (study I) or 1995-2018 (studies II and III) were included. Follow-up started on the day of implantation and finished on the 31st of December 2016 (study I) or the 31st of December 2019 (studies II and III). The endpoint was defined as homograft related death or homograft reintervention, such as surgical replacement of the homograft, endovascular intervention against the PV or other invasive interventions. Data were collected from medical records, the Swedish Registry for Congenital Heart Disease, and the cause of death register of the Swedish National Board of Health and Welfare.

For studies II and III, some reinterventions were redefined as non-valve related, and these patients were censored at reintervention. These interventions included heart transplant, Glenn procedure, total cavopulmonary connection (TCPC) or replacement of the autograft after the Ross procedure. In the cases of heart transplants, patients had complex heart defects where heart failure was the main reason for reintervention rather than homograft failure. In Glenn and TCPC interventions, patients were undergoing surgery at a specific age no matter the homograft function. In one case, the autograft failed in the aortic position after the Ross procedure. It was replaced by a biological valve prosthesis, and the homograft was replaced with the autograft i.e., the native PV.

Patients who died from other causes than homograft complications during follow-up were censored at their time of death. Homograft-related death was defined according to guidelines for reporting mortality and morbidity after cardiac valvular operations (106).

For study III, endocarditis was defined as "definite" or "possible" according to the Duke criteria (107). Positive blood cultures at endocarditis diagnosis were compared to cultures from the homograft preparation to evaluate possible transmission.

Follow-up was conducted at the recipient's domicile hospital. During the first postoperative year, follow-up was conducted every four to sixth months. Later, follow-up was conducted once a year and in some stable cases every second year. Standard postoperative follow-up includes anamnestic interview, examination,

electrocardiography, and echocardiography. Additional investigations were made on indication. Data from the last follow-up was collected.

Decisions on reintervention were made on an individual basis in a multidisciplinary conference. Indications for discussion on reintervention are decreased physical capacity, progressive RV dilatation, RV end-diastolic volume >150 ml/m^2 body surface area, RV ejection fraction <45%, pulmonary regurgitation fraction >40%, PS with maximal gradient of >50 mmHg or >4 m/s on Doppler recording, tricuspid regurgitation, or ventricular arrhythmias.

Mechanical testing (study IV)

The tensile mechanical properties of the samples were tested using a uniaxial testing machine (Instron 8511 load frame, High Wycombe, UK/MTS Test Star II controller, Minneapolis, US). Time, force, and deformation were recorded, and elastic modulus, yield stress and energy at yield stress were calculated. The elastic modulus represents the elasticity of the tissue, where higher values indicate an increased stiffness of the tissue. Yield stress represents the maximum stress (force/cross-sectional area) that can be applied to the tissue before it deforms permanently, thus when the tissue cannot return to its original state. The energy at yield stress describes the total absorbed energy at the point of yield stress.

Tensile tests were performed at room temperature and samples were thawed from cryopreservation just before testing. Routines from thawing in the operating room were imitated, by quick thawing in water at 37°C, before unpacking the samples from their storing bags. Samples were mounted longitudinally in the machine with at least 10 mm distance between the grips (Figure 7).

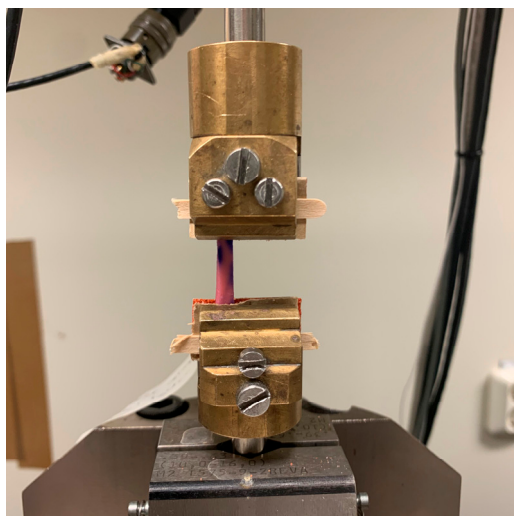


Figure 7. Set-up of mechanical testing.

The sample was stretched manually until no slacking was observed. Thickness and length between the grips of the sample were measured with a digital caliper. From each homograft, three replicate samples per time point were tested.

The specimens were tested until failure at a speed of 1 mm/s and force and displacement data were recorded. The stress was calculated by normalizing the forces with the cross-sectional area. The initial gage length was determined as the length between the grips at 0.05 MPa stress. The strain was then calculated as the displacement divided by this gage length. The elastic modulus was calculated as the slope of the linear part of the stress-strain curve, the yield stress was determined using a 2% offset criterion, and the energy at yield was calculated as the area under the stress-strain curve until the yield point. Mechanical parameters were defined according to Figure 8.

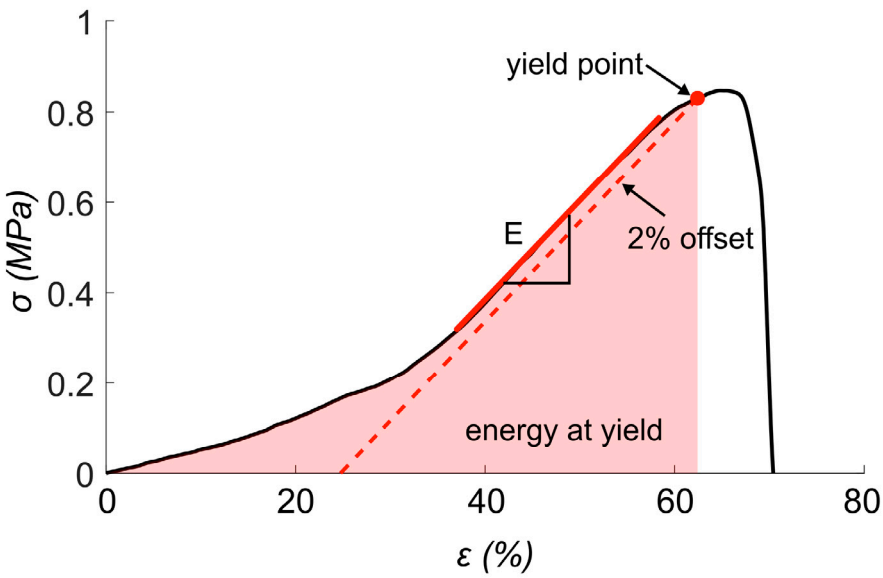


Figure 8. Typical stress-strain curve for a homograft sample, indicating the calculated mechanical properties elastic modulus (E), yield stress and energy at yield stress. Y-axis present stress in megapascal (σ) and x-axis present strain (ε).

Morphological analysis (studies IV and V)

LM and TEM samples were assessed separately with different protocols. Aortic and pulmonary homografts were analyzed in separate groups due to different morphological appearance.

Light microscopy (studies IV and V)

Sample collection and interpretation - Study IV

Homograft samples were assessed with LM to control for cellularity or extracellular matrix changes at different time points. Biopsies (0.5x0.5 cm) were retrieved after mechanical testing from all time points from two different homografts chosen at random. Biopsies were taken from the end of the sample strip that had ruptured during mechanical testing. The chosen homografts served as a random control of the total group to reveal major tissue alterations at prolonged decontamination times. Samples were compared with a control sample where biopsies had been collected from a homograft after two, seven, 28 and 60 days in antibiotic contamination without prior cryopreservation or mechanical testing. Time points for the morphological control samples were chosen since they were available from study V.

Samples were fixed in formalin at 4°C directly after collection. At preparation, samples were dehydrated with increasing concentrations of ethanol and xylene and then embedded in paraffin under vacuum. Sections of 3.5 mm were placed on slides in heated distilled water and then dried overnight in a heating cabinet. Sections were stained with erythrosine saffron for inspection of cell nuclei, elastica van Gieson for elastic fibers and azan for collagen fibers. All samples were scanned with a NanoZoomer-SQ Digital slide scanner (Hamamatsu Photonics, Hamamatsu-city, Japan) and assessed on a computer screen through NDP.view2 Image viewing software (Hamamatsu Photonics, Hamamatsu-city, Japan). Analysis was conducted at 20-40x magnification.

Sample collection and interpretation - Study V

At each time point, three biopsies with a size of 0.5x0.5 cm were retrieved from the homograft vessel wall. Fixation, scanning, and assessment methods were identical to study IV.

Evaluation of LM samples was blinded, the evaluator did not know which day the samples were from. One evaluator assessed all samples. A second evaluator assessed 20% of the samples to validate the protocol and check for inter-rater reliability.

Cells were evaluated with two different categories: cell count and cell nuclei appearance. Three circles with a radius of 100 µm were placed in the media of the vessel wall, one close to the endothelial area, one in the middle of the media, and

one close to the adventitial area. Cell count was calculated as a continuous variable, by counting the total number of cells within each circle (Figure 9A). Cell nuclei appearance was evaluated within the defined circles as well. Cell nuclei appearance was defined as karyolitic, pyknotic or normal in each circle, and given separate score of 1-3 depending on the dominant cell type (Figure 10) (Table 3).

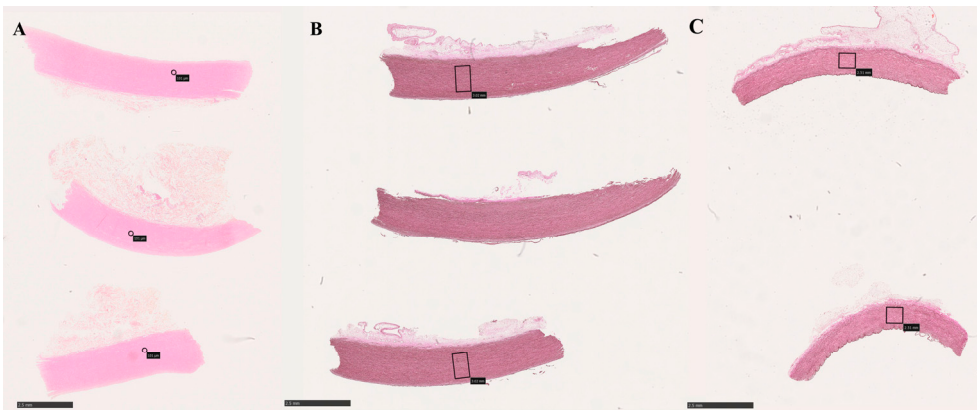


Figure 9. A: Circles used for cell count and cell appearance evaluation. Erythrosine saffron stain, 0.87x magnification. B: Boxes used for elastic fiber evaluation in aortic homografts. C: Boxes used for elastic fiber evaluation in pulmonary homografts. Elastica van Gieson stain, 0.87 magnification.

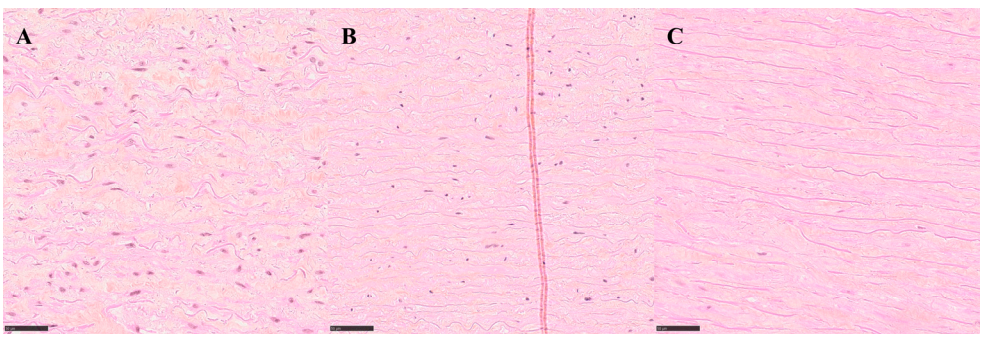


Figure 10. Typical examples of cell appearance. A: Normal cells (day 2) with well-defined nuclei and chromatin. B: Pyknotic cells (day 2) with small, homogenous nuclei. C: Karyolitic cells (day 60) with dissolving nuclei. Erythrosine saffron stain. 40x magnification. Scale bar 50 µm.

Elastin was evaluated as a continuous variable, counting the number of intact elastin fibers. Two areas of 0.8 mm^2 (aortic) or 0.4 mm^2 (pulmonary) were chosen at random for each sample (Figure 9B-C). A smaller area was used for pulmonary homografts since their vessel wall was thinner. Within the area, all intact elastin fibers >100 µm (aortic) or >50 µm (pulmonary) were counted. Different lengths were chosen since the normal aorta has more and longer elastic fibers compared to the normal pulmonary artery. Longer fibers were given a higher score (Table 3).

Collagen was evaluated as a categorical variable. Two separate areas of the sample were evaluated, and each area was given a score from 5 (normal) to 1 (complete destruction) (Table 3) (Figure 11). A summary of the evaluation protocol is shown in Table 3.

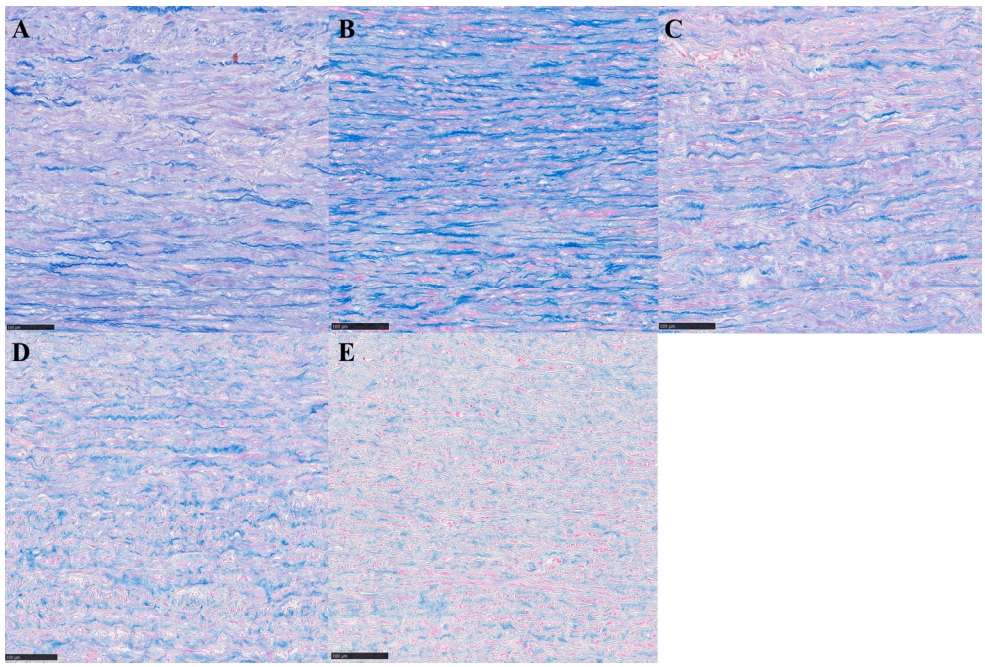


Figure 11. Typical examples of collagen appearance. A: Well-defined, long collagen bundles (day 2), score 5. B: Well-defined, but shorter collagen bundles (day 4), score 4. C: Still visible bundles, but starting to dissolve (day 7), score 3. D: Ill-defined bundled with dissolving fibers (day 21), score 2. E: Collagen pattern completely dissolved, no visible bundles (day 60), score 1. Azan stain, 20x magnification. Scale bar 100 μm.

Table 3. Summary of evaluation protocol for light microscopy.

Structure	Description
Cell Count	The total number of cells found in all three circles.
Cell nuclei appearance	
Score 3	Normal cell nuclei with well-defined nuclei membrane.
Score 2	Pyknotic cells with small, dark, homogenous nuclei.
Score 1	Karyolitic cells with dissolving nuclei and ill-defined nuclei membrane.
Elastin appearance (aortic/pulmonary)	Each intact elastin fiber >100 µm for aortic homografts and >50 µm for pulmonary homografts was given a separate point. Longer fibers generated higher points. Total points were calculated in each defined area.
>100/50 µm	1 point each.
>150/100 µm	2 points each.
>200/150 µm	3 points each.
>250/200 µm	4 points each.
Collagen appearance	
Score 5	Well-defined collagen bundles, with >5 bundles of 75 µm length.
Score 4	Well-defined collagen bundles, but shorter and more fragmented.
Score 3	Less defined bundles that are starting to dissolve.
Score 2	Ill-defined bundles and dissolved fibers, but still intact pattern of collagen within the sample.
Score 1	No visible collagen bundles, no well-defined pattern of collagen within the sample.

Transmission electron microscopy (study V)

At each time point, one biopsy with a size of 0.5x0.1 cm was retrieved from the homograft vessel wall. Biopsies were pre-fixed in 2% paraformaldehyde and 2% glutaraldehyde for 1-24 hours and then rinsed in 0.1 Sorensen's Phosphate Buffer. Samples were fixed with 1% OsO_4 for one hour, followed by dehydration with increasing acetone concentrations. After dehydration, samples were impregnated and embedded in Epon, followed by polymerization in Epon for 48 hours at 60°C. Thin sections of 60 nm were sliced off with a Diatome diamond knife in Leica EM UC7 ultratome and the sections were mounted on Maxtaform H5 formvar coated copper grid. The grids were contrasted with 4% uranylacetat for 20 minutes in 38°C and 1% lead citrate for two minutes at room temperature. Samples were examined with FEI Tecnai biotwin 120KV microscope and images were obtained with an Olympus veleta 2x2k camera.

Transmission electron microscopy interpretation

TEM was used to evaluate ultrastructures of the homograft vessel wall. Evaluation was made by one evaluator without blinding. Five cells, five elastin fibers and five areas with collagen were inspected and evaluated separately within each sample.

Cell appearance was evaluated by chromatin appearance, cell nuclei membrane and cell shrinkage (Figure 12) (Table 4).

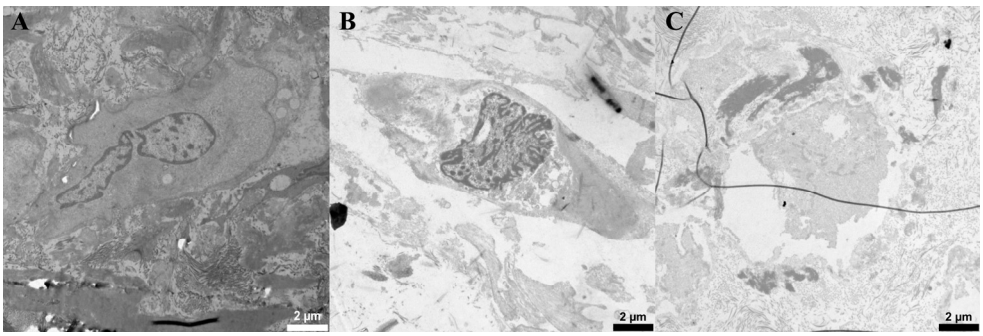


Figure 12. Typical examples of cells in transmission electron microscopy. A: Non-condensed, well-scattered chromatin (score 4) and no cell shrinkage (score 3) (day 0, 6000x magnification). B: Almost fully condensed chromatin appearing as lumps within the nuclei (score 2) and cell shrinkage with the presence of vacuoles (score 1) (day 14, 6000x magnification). C: Completely dissolved nuclei (score 1) with no distinguished chromatin (score 1) and cell shrinkage (score 2) (day 60, 6000x magnification).

Elastin appearance was evaluated by fiber structure and fragmentation, damage within the fibers and the presence of surrounding fibrillin (Figure 13) (Table 4).

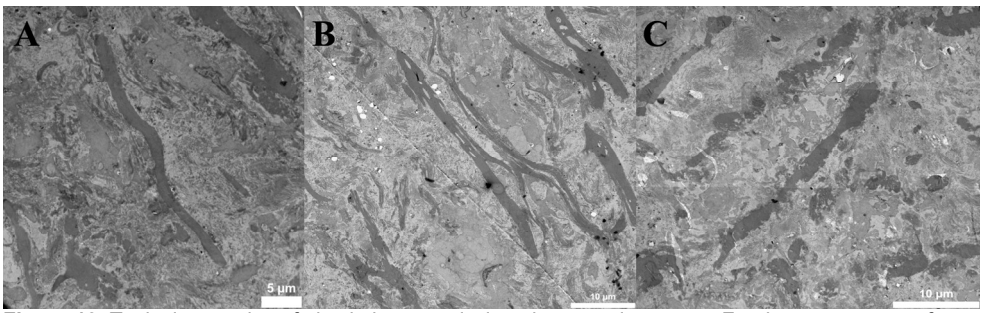


Figure 13. Typical examples of elastin in transmission electron microscopy. For the assessment of fibrillin, a higher magnification was used. A: Well-defined fiber border without damage (score 5), no internal damage (score 3) (day 3, 2550x magnification). B: Partly defined and partly uneven border (score 4), multiple, large areas with internal damage (score 1) (day 4, 6000x magnification). C: Partly uneven border, partly dissolving (score 2), small internal damage (score 2) (day 21, 6000x magnification).

Collagen appearance was evaluated by collagen bundle structure and orientation, shrinkage of bundles, and the presence of a typical D-banding pattern (Figure 14) (Table 4). A summary of the evaluation protocol is shown in Table 4.

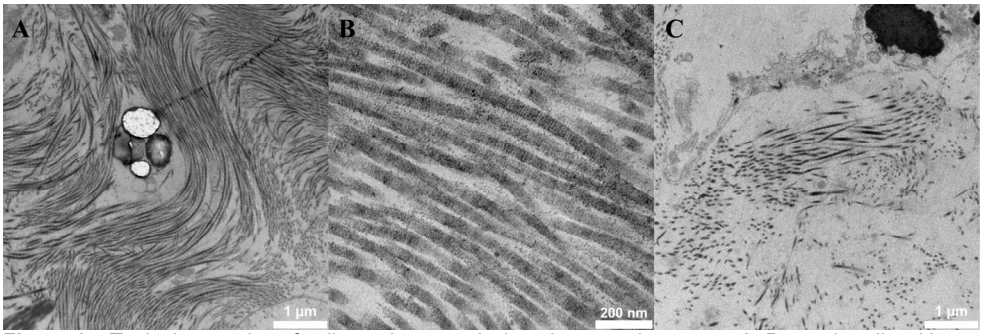


Figure 14. Typical examples of collagen in transmission electron microscopy. A: Dense bundle with parallel fibers (score 3), no shrinkage of bundles (score 2) (day 3, 16500x magnification). B: Clear presence of a D-banding pattern (score 3) (day 28, 87000x magnification). C: Sparse and disoriented fibers within the collagen bundle (score 1), shrinkage of the bundle with loss from surrounding tissue (score 1) (day 60, 16500x magnification).

Table 4. Summary of evaluation protocol for transmission electron microscopy.

Structure	Description		
Cell appearance (total score 3-12)	*Cell nuclei membrane appearance (score 1-5)*	*Cell nuclei chromatin appearance (score 1-4)*	*Cell shrinkage (score 1-3)*
Score 5	Well-defined, double membranes visible.		
Score 4	Well-defined, double membrane only partly visible.	Non-condensed chromatin, well scattered within the cell nuclei.	
Score 3	Well-defined, but only single membrane visible.	Partly condensed chromatin, still scattered within the cell nuclei.	No cell shrinkage
Score 2	Partly defined, partly dissolved.	Fully condensed chromatin, appearing as lumps within the nuclei.	Cell shrinkage, loss from surrounding tissue.
Score 1	Completely dissolved.	Complete dissolved chromatin that cannot be distinguished.	Cell shrinkage and presence of vacuoles.
Elastin appearance (total score 3-10)	*Elastic fiber structure (score 1-5)*	*Elastic fiber internal damage (score 1-3)*	*Presence of surrounding fibrillin (score 1-2)*
Score 5	Well-defined fiber border without damage		
Score 4	Partly-defined fiber border, partly uneven and ruffled.		
Score 3	Uneven, ruffled fiber border.	No internal damage.	
Score 2	Partly uneven fiber border, partly dissolving.	Smaller damage, two or fewer areas per field of view at 6000x magnification.	Presence of surrounding fibrillin.
Score 1	Dissolving fiber.	Single, large damage or multiple areas with damage.	No surrounding fibrillin.
Collagen appearance (total score 3-8)	*Collagen bundle structure and orientation (score 1-3)*	*Presence of typical banding of fibers (score 1-3)*	*Shrinkage of bundles (score 1-2)*
Score 3	Dense bundles with parallel fibers.	Clear presence of D-bands.	
Score 2	Dense bundles but disorientation of fibers within the bundles.	Partly dissolved D-bands.	No shrinkage, bundles lie adjacent to surrounding tissue.
Score 1	Sparse and disoriented fibers within the collagen bundle.	No D-bands.	Shrinkage of bundles, loss from surrounding tissue.

Statistical analysis

Analyses were performed with SPSS 24 software (IBM, Armonk, NY) for study I and Stata (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX, USA: StataCorp LCC) for studies II-V.

Continuous variables are presented as mean or median with standard deviation (SD) or interquartile range (IQR) depending on whether they were normally distributed (mean) or not (median). Categorical variables are presented with absolute numbers and percentages. Differences between continuous variables were analyzed with the Mann-Whitney U-test or the Wilcoxon signed rank test depending on whether the data was paired or not. Differences between proportions in categorical variables were analyzed with the chi^2-test. P-values <0.05 were considered significant unless stated otherwise. The confidence level was set to 95%. There was no correction for multiple testing except for study V.

Studies I-III

In study I, freedom from reintervention was analyzed with the Kaplan-Meier method and differences between groups were analyzed with the log-rank test. The risk factors analyzed were homograft type, position of the homograft, donor age group, donor gender, donor type, ischemic time, and heart retrieval to cryopreservation time. See Table 5 for further definitions of variables. Continuous variables were divided into categorical groups for analysis with the Kaplan-Meier method. According to donor age, groups were set at <1 year, 1-15 years, 16-30 years, 31-50 years and >50 years. The two youngest age groups were chosen because they included the smallest homografts, which are a well-known risk factor for early intervention. The middle age groups were chosen because the median donor age was 30 years. The oldest group was chosen to achieve more equally sized groups. Time limits in ischemic time were set according to commonly used criteria for ischemic time (maximum 24 vs. 48 hours). Time limits for heart retrieval to cryopreservation were set at 30 hours or less, 31-48 hours, 49-120 hours and >120 hours. Some homografts are preserved within a few hours after the minimum 24-hour decontamination time, and a maximum of 30 hours will include most homografts that are cryopreserved the day after retrieval. The 48-hour time limit was set to include homografts that were cryopreserved within two days after retrieval. The 120-hour time limit was set to achieve equally sized groups.

In studies I-III, risk factors were analyzed with univariable and multivariable analysis using Cox proportional hazard regression with homograft reintervention as the dependent variable. For each categorical variable, the group with the lowest proportion of reinterventions was used as the reference group. Risk factors analyzed in different studies are listed in Table 5. Risk factors were chosen because of their clinical importance. Proportional hazard assumptions were checked by graphical

inspection of Kaplan-Meier curves. Median survival was calculated in a survival analysis.

Table 5. Risk factors included in the univariable analysis in studies I-III.

Risk factor	Study I	Study II	Study III	Definition
Homograft type	X	X	X	Aortic or pulmonary
Homograft position	X	X	X	Anatomic or extra-anatomical
Donor age	X	X	X	
Donor gender	X			
Donor type	X	X	X	Non-heart-beating donor, multi-organ donor or domino donor.
Homograft size	X	X	X	
Recipient age	X	X	X	
Ischemic time	X			Time from donor circulatory arrest until heart retrieval
Heart retrieval to cryopreservation time	X			
Time period of surgery		X	X	
Presence of structural changes		X		None, fenestrations or atheromatosis
Microbiological contamination			X	Contamination prior to decontamination (none, low-risk or high-risk)

For study III, microbiological contamination was defined as “none”, “low-risk” or “high-risk”. High-risk microbes were defined as microbes that should lead to immediate discard of tissue according to the EDQM, even if found prior to decontamination (Table 6). Note that this table differs from Table 1 since guidelines were updated in 2022, one year after study III was published (19,108).

Table 6. Contaminants that should result in tissue being discarded if detected at any stage of processing according to European guidelines from the European directorate for the quality of medicine and health care, 2019 (108).

High-risk microbes
Methicillin-resistant *Staphylococcus aureus*
Streptococcus spp. and *Enterococcus* spp.
Clostridium spp.
Salmonella spp., *Shigella* spp., *Klebsiella* spp.
Flavobacterium meningiosepticum
Listeria monocytogenes
Mucor spp.
Mycobacterium spp.
Neisseria gonorrhoeae
Penicillum spp.
Pseduomonas aeruginosa or *Pseudomonas pseudomallai*
Yeast and filamentous fungi (molds).

All variables were considered for inclusion in the multivariable model. To avoid multicollinearity, the correlation between continuous variables was checked with Pearson's test of correlation. A backward stepwise multivariable Cox proportional hazard regression model was conducted. For study I, all variables with a p-value <0.2 in the univariable analysis were included in the multivariable analysis. For studies II and III, all variables except for the variable of interest (presence of structural changes for study II and microbiological contamination for study III) were included in the first multivariable analysis. P-value <0.1 led to stepwise elimination from the model. The variables "presence of structural changes" and "microbiological contamination" were included in the final models for studies II and III respectively since they were the variables of interest for these studies.

Subgroup analysis - study I

In study I, two subgroup analyses were made of the variables ischemic time and homograft size, to further investigate their impact on the time to reintervention.

Ischemic time of 1-24 hours was compared to >24 hours separately (excluding 0 hours) using the Kaplan-Meier method, log-rank test and univariable Cox proportional hazard regression.

Homograft size was analyzed separately with univariable Cox proportional hazard regression, including adult-sized homografts only (>18 mm).

Study IV

Each time point (two to four days, seven to nine days, 28-30 days, and 60-62 days) generated three replicate samples per homograft, which generated three different measurements in the mechanical testing. The mean value of the three measurements was used in the analysis. Day two to four was used as a reference point. Other time points were compared individually to the reference point with a Wilcoxon signed rank test.

Study V

Each time point generated a separate score for LM and TEM. For LM, day 0 was used as a reference point. If data from day 0 was missing, day 1 was used as a reference point. For TEM, day 1 was used as a reference point. Due to practical issues, appropriate material (formaldehyde) could not always be retrieved at day 0 for TEM samples, leading to incomplete sample collection at day 0 for TEM. If data from day 1 was missing as well, day 2 was used as a reference point. All other days were compared separately to the reference points. The score at the reference point was set to 1, and relative differences between groups were calculated, to minimize the impact of differences between homografts. The homografts were used as their own controls, with paired comparisons between the reference point and the following time points for the same homograft. Relative score differences were

analyzed with a Wilcoxon signed rank test for paired data. A post-hoc test for multiple comparisons was conducted with the Bonferroni correction.

Two different evaluators analyzed 20% of the LM sample to validate the assessment protocol. Correlations between the results were analyzed with intraclass correlation for continuous variables and weighted Cohen's Kappa for categorical, ordinal variables.

Summary of main outcomes

Study I	Long-term freedom from reintervention after implantation of homografts in the RVOT, and hazard ratios (HR) for possible risk factors for earlier reintervention related to donor and homograft characteristics and homograft processing methods.
Study II	Long-term freedom from reintervention after implantation of homografts in the RVOT, and HR for the impact of structural impairment of homografts on the risk for earlier reintervention.
Study III	Long-term freedom from reintervention after implantation of homografts in the RVOT, and HR for the impact of microbiological and fungal contamination of homografts before decontamination on the risk for earlier reintervention. Comparison of the prevalence of endocarditis in patients receiving a homograft that had been contaminated before antibiotic decontamination compared to homografts that were negative in all cultures during processing.
Study IV	Absolute differences in mechanical properties (elastic modulus, yield stress and energy at yield stress) between a decontamination time of two to four days compared to longer decontamination intervals (7-60 days).
Study V	Relative differences in score for cells, elastin, and collagen analyzed in LM and TEM between a decontamination time of up to one day and longer decontamination intervals (1-60 days).

Results

Study I

Donors and homografts

During 1995-2008, 304 cryopreserved homografts were distributed from the tissue bank in Lund and used for RVOT implantation at Skane University Hospital. Seven homografts were excluded due to the emigration of recipients shortly after implantation, leaving 297 homografts for inclusion in the analysis. The donor characteristics are summarized in Table 7. The median donor age was 31 years (IQR 13-47, range 0-66). The median homograft size was 20 mm (IQR 17-23, range 9.0-34). The homograft size per donor age group is defined in Table 7. Data were complete for 280 homografts (94%).

Recipients

The recipient characteristics are presented in Table 8. The median age at implantation was 9.2 years (IQR 2.0-15, range 4.0 days-65 years). During the study period, 209 recipients received one homograft, 38 recipients received two homografts and four recipients received three homografts. The follow-up was 99% complete. Two recipients were lost to follow-up and censored at their last follow-up. The median follow-up time was 10 years (IQR 8.0-13, range 0-22 years). The correlation between continuous variables is shown in Table 9.

Table 7. Demographic data on donor characteristics and homograft management.

Variable	Donors (n = 297)
Donor gender	No. (%)
Male	158 (53%)
Female	128 (43%)
Missing	11 (3.7%)
Donor age, years	No. (%)
Infant, 0-0.99	16 (5.4%)
Child, 1-15	69 (23%)
Young adult, 16-30	62 (21%)
Adult, 31-50	90 (30%)
Older adult, >50	60 (20%)
Graft sizes in donor age groups	Median, millimeters (IQR, range)
Infant, 0-0.99	10 (10-12, 9-13)
Child, 1-15	16 (13-18, 9-24)
Young adult, 16-30	20 (18-22, 14-34)
Adult, 31-50	22 (20-24, 16-31)
Older adult, 50	23 (22-25, 19-28)
Donor type	No. (%)
Non-heart-beating donor	173 (58%)
Multi-organ donor	80 (27%)
Domino donor	39 (13%)
Missing	5 (1.7%)
Homograft type	No. (%)
Pulmonary	215 (72%)
Aortic	81 (27%)
Missing	1 (0.34%)
Ischemic time, hours	No. (%)
0	116 (39%)
1-24	54 (18%)
>24	122 (41%)
Missing	5 (1.7%)
Heart retrieval to cryopreservation, hours	No. (%)
≤30	100 (34%)
31-48	46 (16%)
49-120	98 (33%)
>120	47 (16%)
Missing	6 (2.0%)

Table 8. Demographic data on recipients.
IVS = intact ventricular septum, MAPCA = major aortopulmonary collateral artery, PI = pulmonary insufficiency, PS = pulmonary stenosis, TOF = teratology of Fallot, TGA = transposition of the great arteries, VSD = ventricular septal defect.
[a]Others include absent pulmonary valve syndrome, isolated pulmonary stenosis or pulmonary insufficiency, congenital corrected transposition and double-outlet right ventricle with associated pulmonary stenosis or insufficiency.

Variable	Recipients (n = 297)
Recipient gender	No. (%)
Male	166 (56)
Female	131 (44)
Surgical indication	No. (%)
Pulmonary atresia +	
VSD	25 (8.4)
VSD, MAPCA	19 (6.4)
IVS	7 (2.4)
TGA, VSD, PS	8 (2.7)
Truncus arteriosus	28 (9.4)
TOF with postoperative PS or PI	55 (19)
Ross	20 (6.7)
Conduit replacement	95 (32)
Others[a]	40 (14)
Homograft position	No (%)
Extra-anatomic	177 (60)
Anatomic	120 (40)

Table 9. Correlation between continuous variables.
Tested with Pearson's test of correlation. $p<0.01$ for all correlation.

	Recipient age	Donor age	Homograft size
Recipient age		0.53	0.69
Donor age	0.53		0.74
Homograft size	0.69	0.74	

Mortality

Twelve recipients (4.0%) died during follow-up. One death was considered valve-related. The patient died suddenly at home and the autopsy showed rupture of a pseudoaneurysm related to the suture line. The cause of the pseudoaneurysm is unknown but might have been due to surgical technique. Another explanation could be weakness of the muscle cuff of the homograft. The case was closely reviewed, but there were no abnormalities in the collection or processing of the homograft.

All other deaths were considered non-valve-related. There were four early deaths. Two within 30 days caused by coronary events (one infant and one adult). One

within 60 days due to surgical complications leading to cardiac arrest and hypoxic brain damage and one within 90 days due to respiratory failure. There were seven late deaths. Four were cardiac deaths and three were non-cardiac death.

Reintervention

There were 115 (39%) reinterventions during follow-up: 99 patients underwent reoperation with valve replacement, 13 underwent TPVI, two patients underwent heart transplants, and one patient underwent a PV dilatation with the intention to proceed with a Melody valve, but the intervention was interrupted due to a prolonged intervention time.

Freedom from reintervention at 1-, 5-, 10- and 15 years was 99% (95% confidence interval (CI) 98-100%), 90% (95% CI 87-94%), 70% (95% CI 64-75%) and 55% (95% CI 48-62%) respectively.

The Kaplan-Meier method with the log-rank test revealed significantly lower freedom from reintervention for aortic homografts ($p<0.001$), extra-anatomic position ($p<0.001$), young donor age ($p<0.001$, Figure 15), male gender ($p = 0.018$), NHBD ($p = 0.003$, Figure 16), ischemic time 1-24 hours ($p<0.001$, Figure 17) and retrieval to cryopreservation time ≤30 hours ($p = 0.03$). The median survival within each variable is presented in Table 10.

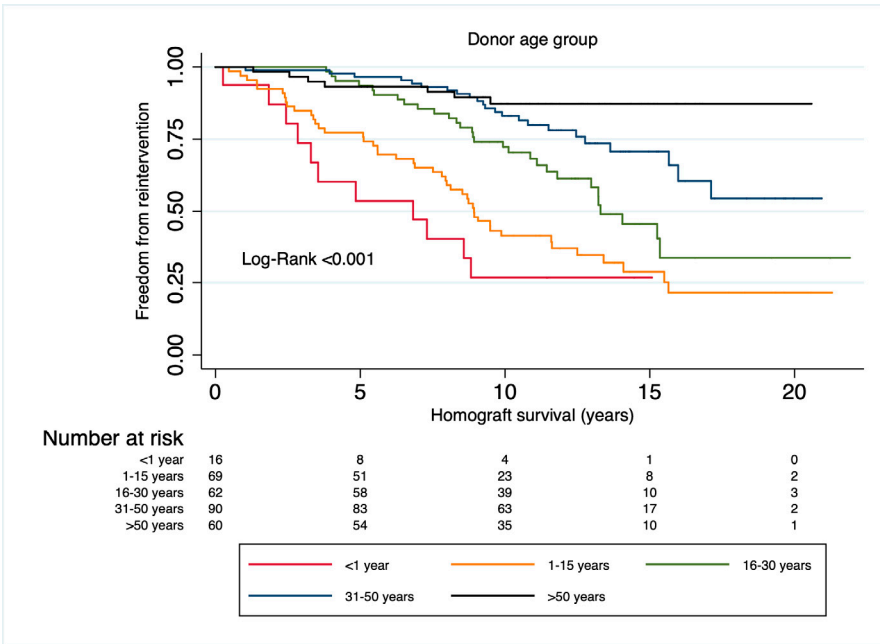


Figure 15. Kaplan-Meier analysis with the log-rank test of donor age group.

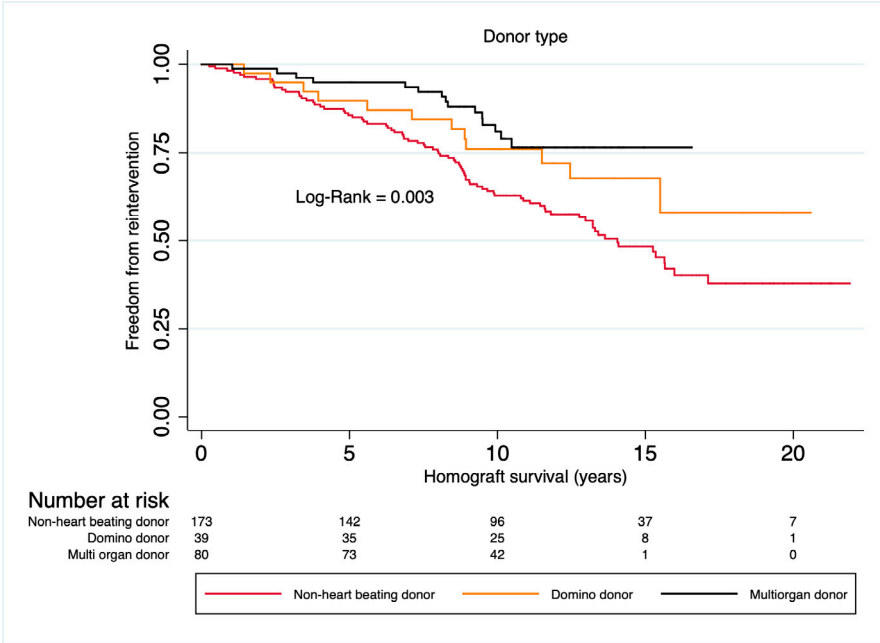


Figure 16. Kaplan-Meier analysis with the log-rank test of donor type.

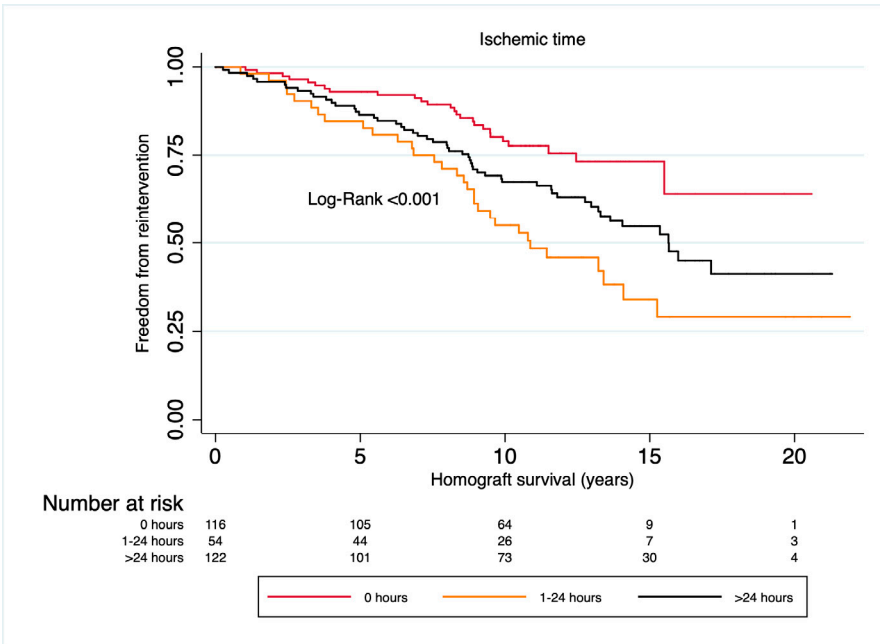


Figure 17. Kaplan-Meier analysis with the log-rank test of ischemic time group.

The results from the univariable and multivariable analysis are presented in Table 10. Multivariable analysis showed that aortic homografts, young donor age, and ischemic time of 1-24 hours are risk factors for early reintervention.

Table 10. Results from analysis of freedom from reintervention.
Univariable and multivariable analysis with Cox proportional hazard regression. The group with the lowest proportion of reinterventions is used as a reference group for each variable. CI = confidence interval. HR = hazard ratio. N/A = not available. NHBD = non-heart-beating donor. *Significant result ($p<0.05$).

Variable	Median time to reintervention (years)	Univariable analysis			Multivariable analysis (n = 280)		
		HR	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Homograft type							
Aortic	11	1.7	1.9-3.9	<0.001*	1.9	1.3-2.8	0.0020*
Pulmonary	20	1.0			1.0		
Homograft position							
Anatomic	17	1.0			1.0		
Extra-anatomic	14	2.4	1.6-3-7	<0.001*	1.0	0.60-1.8	0.87
Donor age, years	N/A	0.96	0.95-0.97	<0.001*	0.97	0.95-0.98	<0.001*
Donor gender							
Male	15	1.6	1.1-2.4	0.022*	1.5	1.0-2.3	0.050
Female	20	1.0			1.0		
Donor type							
NHBD	15	2.4	1.4-4.1	0.0020*	0.95	0.13-7.2	0.96
Domino donor	20	1.4	0.70-3.0	0.37	0.54	0.24-1.2	0.14
Multi-organ donor	20	1.0			1.0		
Homograft size, mm	N/A	0.84	0.81-0.88	<0.001*	0.95	0.88-1.0	0.20
Recipient age, years	N/A	0.93	0.90-0.95	<0.001*	1.0	0.97-1.0	0.79
Ischemic time							
0 hours	20	1.0			1.0		
1-24 hours	11	2.7	1.6-4.6	<0.001*	1.7	1.0-3.0	0.043*
>24 hours	16	1.8	1.1-2.9	0.017*	1.1	0.67-1.8	0.74
Retrieval to cryopreservation time							
≤30 hours	14	1.7	1.1-2.7	0.024*	1.1	0.62-1.9	0.80
31-48 hours	20	0.9	0.5-1.7	0.78	1.0	0.53-2.1	0.90
49-120 hours	20	1.0			1.0		
>120 hours	20	1.0	0.50-1.9	0.98	1.3	0.64-2.5	0.51

Subgroup analysis of ischemic time

A separate analysis of 1-24 hours of ischemic time before heart retrieval compared to >24 hours was conducted. Demographic data of the subgroups are described in Table 11. The recipient age was significantly younger in the group with 1-24 hours of ischemic time. Kaplan-Meier analysis with a log-rank test was borderline significant (p=0.05) with a lower freedom from reintervention in the group with 1-24 hours of ischemic time. Univariable analysis with Cox proportional hazard regression showed no significant differences between the groups (HR 1.5, 95% CI 0.99-2.4, p = 0.058).

Table 11. Demographic data of the groups with ischemic time 1-24 hours and >24 hours. [a]Mann-Whitney U-test. [b]Chi^2-test

	Ischemic time 1-24 hours (n = 54)	Ischemic time >24 hours (n = 122)	P-value
Median age			
Recipients (years)	4.0	9.0	0.033[a]
Donors (years)	22	25	0.29[a]
Aortic homografts, No. (%)	18 (33%)	41 (34%)	0.94[b]
Male donor, No. (%)	29 (54%)	80 (66%)	0.13[b]
Median size (mm)	18	19	0.17[a]

Subgroup analysis of donor age

Analysis of donor age was conducted when including adult-sized homografts only (>18 mm). This group included 189 homografts with a median donor age of 42 years (IQR 27-52, range 7-66 years). Ten donors were aged 17 years or younger. Univariable analysis with Cox proportional hazard regression showed a significant result with a higher reintervention rate in recipients of homografts from a younger donor, although excluding small homografts (HR 0.96, 95% CI 0.95-0.98, p<0.001).

Study II

Donors and homografts

During 1995-2018, 2840 homografts were collected from the tissue bank in Lund. Of all homografts, 1281 (45%) were discarded and 534 (19%) were implanted in the RVOT at Skane University Hospital in Lund. Of the 534 implanted homografts, 19 were excluded due to recipient migration shortly after surgery and six were excluded because they were implanted after the 31st of December 2018, leaving 509 homografts included in the analysis. Donor and homograft characteristics are presented in Table 12. Homografts that were neither discarded nor implanted in the RVOT were used for LVOT implantation (n = 167), sent to another hospital (n = 659), used for patch implantation (n = 15), were still in the bank at the last follow-

up (n = 42) or were missing a report of destination (n = 30). Missing data is due to incomplete registration of homografts.

Table 12. Donor and homograft characteristics.
"All" refers to all collected homografts. NHBD = non-heart-beating donor.

	All		Discarded		Implanted for RVOT, Lund	
	n=2860	*%*	*n=1281*	*%*	*n=509*	*%*
Donor age, years						
0-1	129	4.5	36	2.8	29	5.7
1-15	202	7.1	38	3.0	88	17
15-30	564	20	205	16	141	28
30-50	888	31	404	32	135	27
≥50	1075	38	588	46	116	23
Missing	2	0.070	2	1.6		
Donor gender						
Male	1680	59	796	63	292	57
Female	1152	40	462	36	213	42
Missing	28	2.0	15	1.2	4	0.79
Donor type						
Multi-organ donor	1119	39	492	39	151	30
Domino donor	461	16	249	20	63	12
NHBD, 1-24 hours	495	17	193	15	112	22
NHBD, >24 hours	767	27	321	25	183	36
Missing	18	0.63	18	1.4		
Homograft type						
Pulmonary	1440	50	580	46	417	82
Aortic	1420	50	693	54	92	18
Homograft size, mm						
0-10	101	3.5	35	2.7	20	3.9
10-20	519	18	104	8.2	169	33
20-30	1522	53	418	33	320	63
≥30	12	0.42	10	0.78		
Missing	706	25	706	56		
Ischemic time, hours						
0	1582	55	743	58	214	42
1-24	484	17	184	15	111	22
>24	770	27	322	25	184	36
Missing	24	0.84	24	1.9		

The main reasons for discard were structural changes such as fenestrations of the cusps (43%) or atheromatosis (25%). All discard reasons are presented in Table 13. The prevalence of structural changes in the homografts is presented in Table 14. More than one structural change can be present in the same homograft. Data

on structural changes are often missing in discarded homografts. Often, the main reason for discard is stated and other impairments are not registered. Fenestration location was often missing in the registries, and fenestration size was often described as “minimal”, “small” or “large” with no exact measurements.

Table 13. Reason for homograft discard.

Homograft discard reason	*n = 1281*	
Fenestration	542	42%
Atheromatosis	322	25%
Serology	74	5.8%
Preparation damage	58	4.5%
Thawed at surgery but not used	57	4.5%
Unacceptable large diameter	50	3.9%
Microbiology	37	2.9%
Cryopreservation tank breakdown	37	2.9%
Histological contraindications	23	1.8%
Lack of space in bank	19	1.5%
Donor contraindication	18	1.4%
Passed expiration date	17	1.3%
Bad tissue quality at preparation	7	0.55%
Valve leakage	3	0.23%
Missing	17	1.3%

Table 14. Presence of structural changes in homografts.
“All” refers to all collected homografts. [a]One homograft with vegetation and one homograft with fused cusps. [b]One homograft with an aneurysm.

	All		Discarded		Implanted for RVOT, Lund	
Cusps	*n = 2860*	*%*	*n = 1281*	*%*	*n = 509*	*%*
No impairment	1620	57	282	22	471	93
Fenestration	727	25	592	47	31	6.1
Fibrosis	203	7.1	90	7.1	7	1.4
Prolapse	7	0.24	7	0.55		
Other[a]	2	0.070	2	0.16		
Missing	301	11	300	24		
Atheromatosis	*n = 2860*	*%*	*n = 1281*	*%*	*n = 509*	*%*
No	1754	61	343	27	502	98
Yes	481	17	396	31	7	1.4
Other[b]	1	0.035	1	0.078		
Missing	624	22	533	42		
Fenestration type	*n = 727*	*%*	*n = 592*	*%*	*n = 31*	*%*
Central	39	5.4	34	5.7	0	0
Peripheral	120	17	24	4.1	22	71
Missing	568	78	534	90	9	29

Recipients

There were 440 recipients receiving a total of 509 included homografts during follow-up. Recipient characteristics are presented in Table 15. The median age at implantation was 13 years (IQR 4.9-25, range 0-72 years). The median follow-up was 9.9 years (IQR 4.8-15, range 10 days-24 years). The follow-up was 98% complete.

Table 15. Recipient characteristics.
IVS = intact ventricular septal defect, MAPCA = Major aortopulmonary collateral artery, PI = Pulmonary insufficiency, PS = Pulmonary stenosis, TOF = Teratology of Fallot, TGA = Transposition of the great arteries, VSD = Ventricular septal defect. [a]Other include absent pulmonary valve syndrome, isolated pulmonary insufficiency or stenosis, congenital corrected transposition, and double-outlet right ventricle with associated pulmonary insufficiency or stenosis.

Variable	Recipients *(n = 509)*	%
Recipient age, years		
0-1	55	11
1-7	101	20
7-18	206	40
≥18	147	29
Diagnosis		
Pulmonary atresia +		
VSD	18	3.5
VSD, MAPCA	23	4.5
IVS	12	2.4
TGA, VSD, PS	9	1.8
Truncus arteriosus	34	6.7
TOF with postoperative PS or PI	118	23
Ross	81	16
Conduit exchange	154	30
Other[a]	60	12
Gender		
Male	300	59
Female	209	41
Anatomic position		
Anatomic	286	56
Extra-anatomic	223	43
Conduit number		
First	355	70
Second	118	23
Third	30	5.9
Forth	5	1.0
Sixth	1	0.20
Time period of surgery		
1995-2002	143	28
2003-2010	237	47
2011-2018	129	26

Mortality

Twenty-four (5.5%) recipients died during follow-up. One death was considered valve-related; see the result from study I that describes this case.

All other deaths were considered non-valve-related. There were six early deaths. Two within 30 days caused by a coronary event (n = 1) and surgical complications (n = 1); one within 60 days due to cardiac arrest with hypoxic brain damage; three within 90 days due to heart failure (n = 1), respiratory failure (n = 1) and a coronary event (n = 1). There were 17 late deaths. Nine patients died from cardiac events (myocardial infarction, heart failure, arrhythmia, or cardiac arrest). Five patients died from malignancy. Two patients died from gastrointestinal diseases. One patient died from unknown causes.

Reintervention

There were 136 (27%) reinterventions during follow-up: 110 patients underwent reoperation with valve replacement using a homograft (n = 79), a Contegra valve (n = 23) or another biological heart valve (n = 8), 25 underwent endovascular reintervention with TPVI (n = 18), balloon dilation (n = 4) or stent insertion (n = 3), and one underwent reoperation with resection of adherences affecting the homograft. Eight patients underwent reinterventions that were considered non-homograft-related, including five heart transplants, one Glenn procedure, one TCPC and one patient who had his native PV reimplanted in the RVOT a few days after a Ross intervention due to dysfunction in the aortic position. The autograft was replaced by a biological valve in the aortic position.

The median time to reintervention was 7.5 years (IQR 3.3-10, range 84 days-23 years). Freedom from reintervention at 1-, 5-, 10-, 15- and 20 years was 98% (95% CI 96-99%), 89% (95% CI 86-92%), 76% (95% CI 71-80%), 67% (95% CI 62-72%), and 57% (95% CI 50-64%) respectively.

The results from the univariable and multivariable analyses are presented in Table 16. Donor age was excluded in the multivariable analysis due to a high correlation to homograft size (0.71, Pearson's test of correlation). Multivariable analysis showed a small homograft size and young patient age as risk factors for early reintervention. Structural impairment of the homograft showed no impact on the reintervention rate.

Table 16. Results of analysis of freedom from reintervention.
Univariable and multivariable analysis with Cox proportional hazard regression. The group with the lowest proportion of reinterventions is used as a reference group for each variable. CI = confidence interval. HR = hazard ratio. NHBD = non-heart-beating donor. *Significant results (p<0.05).

	Univariable analysis			Multivariable analysis (n = 280)		
Variable	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Structural changes						
No changes	1.0			1.0		
Fenestrations	0.27	0.070-1.1	0.068	0.46	0.11-1.9	0.28
Fibrosus or atheromatosis	0.88	0.28-2.8	0.83	0.80	0.25-2.6	0.70
Homograft type						
Pulmonary	1.0					
Aortic	4.17	3.0-5.9	<0.001*	1.5	0.99-2.2	0.053
Homograft size, mm	0.80	0.77-0.83	<0.001*	0.88	0.83-0.94	<0.001*
Donor age, years	0.94	0.93-0.95	<0.001*			
Donor type						
Multi-organ donor	1.0					
Domino donor	1.9	1.0-3.6	0.042*			
NHBD 1-24 hours	2.9	1.7-4.9	<0.001*			
NHBD >24 hours	2.0	1.2-3.3	0.0070*			
Homograft position						
Anatomic	1.0					
Extra-anatomic	4.1	2.8-6.1	<0.001*			
Recipient age, years	0.90	0.88-0.92	<0.001*	0.95	9.93-0.98	0.0010*
Time period of surgery						
1995-2002	1.1	0.60-2.1	0.71			
2003-2010	0.87	0.47-1.6	0.66			
2011-2018	1.0					

Study III

Donors and homografts

The homograft cohort is the same as used in study II. In summary, 2840 homografts were collected during 1995-2018. There were 1281 discards and 534 RVOT implantations, where 509 were included in the analysis (see page 59). Donor and homograft characteristics for implanted homografts are presented in Table 12. Microbiological contamination is presented in Table 17.

Table 17. Donor and homograft characteristics.
IQR = interquartile range. NHBD = non-heart-beating donor.

Variable	*Median/n*	*IQR/%*	*Range*
Microbiological contamination pre-decontamination			
No	351	69	
Low-risk microbe	123	24	
High-risk microbe	35	6.8	

Four homografts were discarded due to positive cultures prior to decontamination, namely three cases of Pseudomonas spp. and one case of extensive microbiological growth detected during the morphological evaluation.

Contamination

The amount of contaminated homografts during different steps of the collection process is described in Figure 18.

Of 690 contaminated homografts, 499 (72%) were contaminated with a single microbe and 191 (28%) were contaminated with two or more microbes. There were 140 (20%) homografts that were contaminated with at least one high-risk microbe (Table 6).

Contamination prior to antibiotic decontamination was significantly more frequent in NHBD (44%) compared to MOD (20%) and domino donors (17%), $p<0.001$ (chi^2-test). The difference was not significant after decontamination of the homografts (2.4% in NHBD, 1.3% in MOD and 1.9% in domino donors, $p = 0.40$, chi^2-test). There was no significant difference in the contamination rate prior to decontamination when comparing NHBD with an ischemic time of 1-24 hours (42%) compared to >24 hours (45%) ($p = 0.32$, chi^2-test).

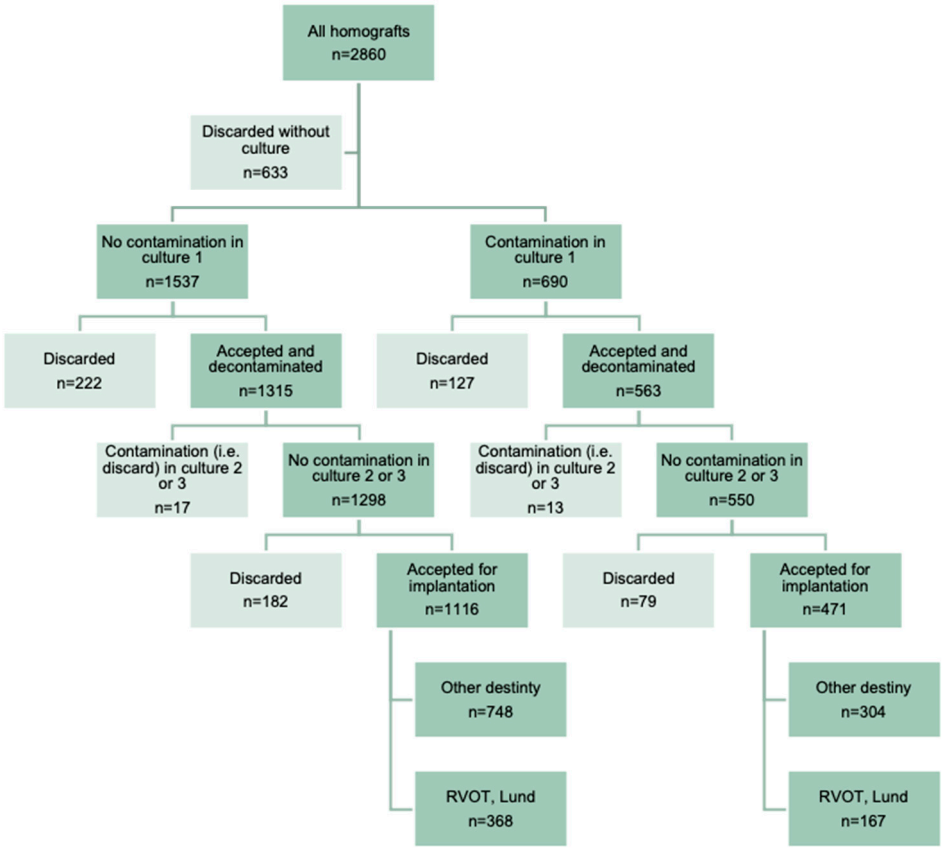


Figure 18. Flow-chart of homograft destiny and amount of contaminated homografts at different steps in the collection process. RVOT = Right ventricular outflow tract.

There were 1537 homografts with negative cultures prior to decontamination, where 16 (1.0%) had positive cultures after decontamination. Thirteen were positive in cultures from tissue or cryoprotective fluid taken prior to cryopreservation, thus leading to the discard of the homografts, and three were positive in cultures from tissue retrieved at implantation of the homograft (see recipients for further details).

Types of microbes that presented in the cultures are presented in Table 18. Some species are only defined by gram-positive status and shape (rod or cocci); these could not be further defined in the cultures. Some homografts were contaminated with more than one microbe.

Table 18. Microbes presenting prior to and after antibiotic decontamination.
[a]Other refers to low-risk microbes constituting <2.0% of the total contamination and only occurring pre-decontamination.

Microbe or fungi	All collected homografts with culture growth			
	Prior to decontamination		Post decontamination	
	n=690	*%*	*n=33*	*%*
Cutibacterium acnes	247	36	6	18
Alpha *streptococcus*	212	31	5	15
Coagulase-negative *staphylococci*	128	19	1	3.0
Clostridium spp.	61	8.8	2	6.1
Staphylococcus aureus	43	6.2		
Corynebacterium spp.	31	4.5	4	12
G+ cocci	27	3.9		
Enterococcus spp.	21	3.0		
Streptococcus spp.	18	2.6		
Group B streptococcus	14	2.0		
Candida spp.	9	1.3	5	15
Pseudomonas aeruginosa	9	1.3	2	6.1
G- rod	6	0.87	2	6.1
Klebsiella oxytoca	5	0.72		
G+ rod	3	0.43	1	3.0
Neisseria spp.	2	0.29		
Aspergillus	2	0.29	2	6.1
Serratia spp.	2	0.29	1	3.0
Stenotrophomonas maltophilia	2	0.29	2	6.1
Flavobacterium spp.	1	0.14		
Other[a]	91	13		
Missing.	5	0.72	2	6.1

Recipients

Details on recipients are described on page 65 and in Table 15. Of 509 homografts, 158 (31%) were contaminated prior to decontamination. Recipient characteristics among contaminated and non-contaminated homografts are presented in Table 20.

Three (0.59%) homografts had a positive culture at implantation. The first case was a one-year-old boy who underwent the Ross procedure. All previous cultures had been negative, but the culture at implantation showed growth of a gram-positive rod. Postoperatively, antibiotic treatment was prolonged due to pleural effusions. He was discharged after nine days. At the last follow-up, five years had passed, and the homograft showed good function.

The second case was a 12-year-old girl who underwent a conduit replacement. Homograft cultures prior to antibiotic decontamination were positive for *Cutibacterium acnes*, but cultures after decontamination were negative. The culture at implantation was positive for *Candida albicans*. Postoperatively, the girl had a normal recovery. The homograft was explanted after 13 years due to stenosis.

The third case was a 13-year-old boy who underwent a conduit replacement. Homograft cultures prior to antibiotic decontamination were positive for *Streptococcus anginosus*, but cultures after decontamination were negative. The culture at implantation was positive for *Aerococcus viridans*. Postoperatively, antibiotic treatment was prolonged due to fever, diarrhea, and high levels of C-reactive protein. Blood cultures were negative, but feces cultures were positive for *Clostridium difficile*. He was discharged after 12 days without signs of infection. Cultures from the homograft did not come back until after the patient was discharged. At that point, an infection specialist was consulted, and the boy received 14 days of ampicillin treatment orally as a safety measure. The patient recovered well. At the last follow-up, three years had passed, and the homograft had a mild stenosis without indication for reintervention.

Mortality

Twenty-four (5.5%) recipients died during follow-up. The patient cohort is the same as in study II and the causes of mortality are described above (page 66).

Endocarditis

There were 18 (3.5%) cases of endocarditis during follow-up and six cases required surgical intervention (Table 20). There was no difference in the proportion of endocarditis between non-contaminated and previously contaminated homografts ($p = 0.83$, chi^2-test).

Table 19. Recipient characteristics.
IQR = interquartile range. IVS = intact ventricular septum, MAPCA = Major aortopulmonary collateral artery, PI = Pulmonary insufficiency, PS = Pulmonary stenosis, TOF = Teratology of Fallot, TGA = Transposition of the great arteries, VSD = Ventricular septal defect. [a]Other include absent pulmonary valve syndrome, isolated pulmonary insufficiency or stenosis, congenital corrected transposition and double-outlet right ventricle with associated pulmonary insufficiency or stenosis.

	All recipients (n = 509)		No contamination (n = 351)		Low-risk contamination (n = 123)		High-risk contamination (n = 35)	
Variables	*Median/n*	*IQR/%*	*Median/n*	*IQR/%*	*Median/n*	*IQR/%*	*Median/n*	*IQR/%*
Recipient age, years	13	4.9-24	12	5.0-22	15	7.9-27	4.6	1.4-17
Diagnosis								
Pulmonary atresia, VSD	18	3.5	12	3.4	3	2.4	3	8.6
Pulmonary atresia, VSD, MAPCA	23	4.5	15	4.3	5	4.1	3	8.6
TGA, VSD, PS	9	1.8	5	1.4	2	1.6	2	5.7
Truncus arteriosus	34	6.7	25	7.1	3	2.4	6	17
Pulmonary atresia, IVS	12	2.4	11	3.1	0	0	1	2.9
Conduit exchange	154	30	30	41	33	8	8	23
TOF with postoperative PS or PI	118	23	86	25	31	25	1	2.9
Ross	81	16	51	15	26	21	4	11
Other[a]	60	12	41	12	12	9.8	7	20
Gender								
Male	300	59	210	60	77	63	13	37
Anatomic position								
Anatomic	286	56	200	57	74	60	12	34
Extra-anatomic	223	43	151	43	49	40	23	66
Conduit number								
First	355	70	246	70	82	67	27	77
Second	118	23	83	24	29	24	6	17
Third and more	36	7.1	22	6.3	12	9.8	2	5.7
Time period of surgery								
1995-2002	143	28	80	23	43	35	20	57
2003-2010	237	47	179	51	48	39	10	29
2011-2018	129	25	92	26	32	26	5	14

Table 20. Proportion of endocarditis during follow-up.
[a]According to Duke criteria (107).

	No contamination			Contamination prior to decontamination		
	n=351	*%*	*Median time to onset (years)*	*n=158*	*%*	*Median time to onset (years)*
No endocarditis	336	97		151	96	
Endocarditis	12	3.4	8.4 (5.7-13)	6	3.8	5.2 (2.2-13)
Possible[a]	6	1.7	5.7 (1.1-11)	4	2.5	5.2 (2.3-10)
Definite[a]	6	1.7	9.9 (7.0-15)	2	1.3	10 (2.1-19)

Among recipients of homografts that were contaminated prior to decontamination, there were six cases of endocarditis. All cases were closely reviewed for signs of possible transmission from the homograft. The results are summarized in Table 21.

There were two cases (cases 2 and 4) where the homograft had positive cultures with high-risk microbes (clostridium spp. and enterococcus spp.) prior to decontamination. Blood cultures at endocarditis diagnosis showed neither of these bacteria. Two cases (cases 3 and 6) showed the same type of microbes at endocarditis diagnosis as the homograft had prior to decontamination (alfa-streptococcus in both cases). Case 3 was a nine-year-old recipient undergoing conduit replacement. Endocarditis occurred 19 years after implantation and was treated with surgical intervention with implantation of a new homograft. Case 6 was an 18-year-old recipient undergoing a primary reconstruction. Endocarditis occurred two years and three months after implantation and was treated successfully with antibiotics. At the last follow-up, five years had passed since the endocarditis diagnosis and the homograft showed good function.

Table 21. Cases of endocarditis among recipients of a homograft that was contaminated during processing, before antibiotic decontamination.
[a]According to Duke criteria (107). [b]Positive blood cultures at endocarditis diagnosis. [c]Positive cultures during the homograft process.

	Time from implantation (years)	Definition[a]	Microbe endocarditis[b]	Microbe homograft[c]	Treatment
Case 1	13	Possible	Coagulase negative *Staphylococcus*	Gram-positive cocci.	Conservative
Case 2	8.1	Possible	*Staphylococcus*	*Clostridium* spp. and *enterococcus* spp.	Conservative
Case 3	19	Definite	*Streptococcus mitis*	*Alfa-streptococcus*	Surgery
Case 4	2.4	Possible	*Streptococcus mitis*	*Clostridium* spp.	Conservative
Case 5	2.1	Definite	*Staphylococcus Aureus*	*Alfa-streptococcus* and coagulase negative *Staphylococcus.*	Surgery
Case 6	2.2	Possible	*Alfa-streptococcus*	*Alfa-streptococcus*	Conservative

Reintervention

There were 136 (27%) reinterventions during the follow-up. The patient cohort is the same as in study II and details on reinterventions are described above (page 66). The results from the univariable and multivariable analyses are presented in Table 22. Donor age was excluded in the multivariable analysis due to a high correlation to homograft size (0.71, Pearson's test of correlation). Multivariable analysis confirmed small homograft size and young patient age as risk factors for early reintervention. Contamination was not a significant risk factor in the multivariable model.

Table 22. Results from analysis of freedom from reintervention.
Univariable and multivariable analysis with Cox proportional hazard regression. The group with the lowest proportion of reinterventions is used as a reference group for each variable. CI = confidence interval. HR = hazard ratio. NHBD = non-heart-beating donor. *Significant result (p<0.05)

	Univariable analysis			Multivariable analysis		
Variable	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Microbiological contamination pre-decontamination						
No	1.3	0.82-1.9	0.28	1.1	0.73-1.7	0.58
Low-risk microbe	1.0			1.0		
High-risk microbe	2.8	1.6-5.1	<0.001*	1.6	0.87-2.8	0.13
Homograft type						
Pulmonary	1.0					
Aortic	4.1	2.9-5.8	<0.001*	1.4	0.96-2.1	0.081
Homograft size, mm	0.80	0.77-0.83	<0.001*	0.88	0.83-0.93	<0.001*
Donor age, years	0.94	0.93-0.95	<0.001*			
Donor type						
Multi-organ donor	1.0					
Domino donor	1.9	1.0-3.6	0.041*			
NHBD 1-24 hours	2.9	1.7-4.9	<0.001*			
NHBD >24 hours	2.0	1.2-3.3	0.0070*			
Homograft position						
Anatomic	1.0					
Extra-anatomic	4.1	2.8-6.0	<0.001*			
Recipient age, years	0.90	0.88-0.92	<0.001*	0.96	0.93-0.98	0.0020*
Time period of surgery						
1995-2002	1.1	0.61-2.1	0.68			
2003-2010	0.86	0.47-1.6	0.64			
2011-2018	1.0					

Study IV

Homografts

Ten aortic homografts were collected during 2020-2021. The mean donor age was 43 years (SD 12, range 20-69). There were three MOD (30%) and seven NHBD (70%). The mean ischemic time for NHBD was 30 hours (SD 10, range 11-42 hours).

Mechanical testing

A total of 120 samples were tested. Fourteen (12%) samples had missing data due to problems during measurements. The remaining 106 samples were included in the analysis. The length, width, and thickness of the sample were measured prior to testing. The results are presented in Table 23.

The results from the mechanical testing are presented in Table 24 and Figure 19. Elastic modulus, yield stress and energy at yield stress reached their highest values in the reference group of two to four days of antibiotic decontamination prior to cryopreservation. Elastic modulus was significantly lower in all other groups with a prolonged decontamination time, showing that the stiffness of the tissue had decreased at time intervals of seven to nine days and beyond (Table 24, Figure 19A). Yield stress was significantly lower at seven to nine and 28-30 days, but not significant at 60-62 days (Table 24, Figure 19B). Energy at yield stress showed no significant differences at prolonged times (Table 24, Figure 19C)

Table 23. Measurements of samples at different times.
IQR = interquartile range. *Significant p-value <0.05. Differences were analyzed with Wilcoxon signed rank test. All groups are compared separately to days two to four.

	Days 2-4		Days 7-9			Days 28-30			Days 60-62		
Measurement in mm	*Median*	*IQR*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
Length	22	17-28	18	17-21	0.26	18	14-25	0.013*	16	14-20	0.037*
Width	5.1	4.7-5.5	4.8	4.6-5.2	0.36	4.9	4.6-5.2	0.20	4.7	4.5-5.1	0.037*
Thickness	1.7	1.6-2.0	1.6	1.5-1.8	0.61	1.6	1.4-1.8	0.037*	1.6	1.4-1.9	0.041*

Table 24. Results from uniaxial tensile testing.
IQR = interquartile range. MPa = megapascal. mJ/mm^3 = millijoule/square millimeter. *p-value <0.05. Differences were analyzed with the Wilcoxon signed rank test. All groups are compared separately to days two to four.

	Days 2-4		Days 7-9			Days 28-30			Days 60-62		
Variable	*Median*	*IQR*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
Elastic modulus (MPa)	2.7	2.5-5.0	1.8	1.4-2.3	0.0080*	2.2	2.0-2.6	0.0080*	2.4	2.1-2.5	0.015*
Yield stress (MPa)	0.78	0.68-1.0	0.53	0.47-0.69	0.028*	0.73	0.57-0.80	0.028*	0.71	0.68-0.84	0.37
Energy at yield stress (mJ/mm^3)	0.16	0.13-0.19	0.11	0.067-0.15	0.051	0.16	0.095-0.19	0.37	0.14	0.12-0.20	0.86

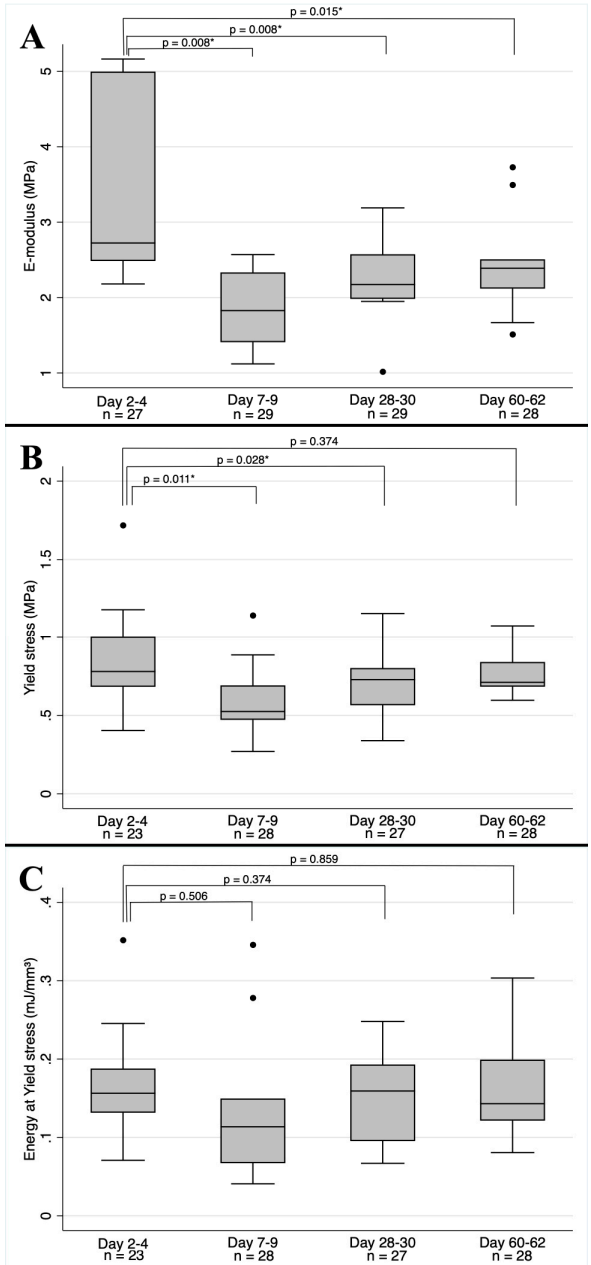


Figure 19. A: Elastic modulus at different times. B: Yield stress at different times. C: Energy at yield stress at different times.
The middle line is the median, the upper and lower axes correspond to interquartile range (IQR). The upper and lower whiskers extend from the axis to the largest and smallest value no further than 1.5x IQR from the axis. Data beyond whiskers are outliers that are plotted individually. *Significant result ($p<0.05$). Differences between groups are calculated with the Wilcoxon signed rank test.

Morphological interpretation

There were no differences in the appearance of cell nuclei between two to four days and seven to nine days. Sample 1 showed no differences in 28-30 days or 60-62 days either, but sample 2 showed almost complete acellularity at days 28-30 and beyond (Figure 20).

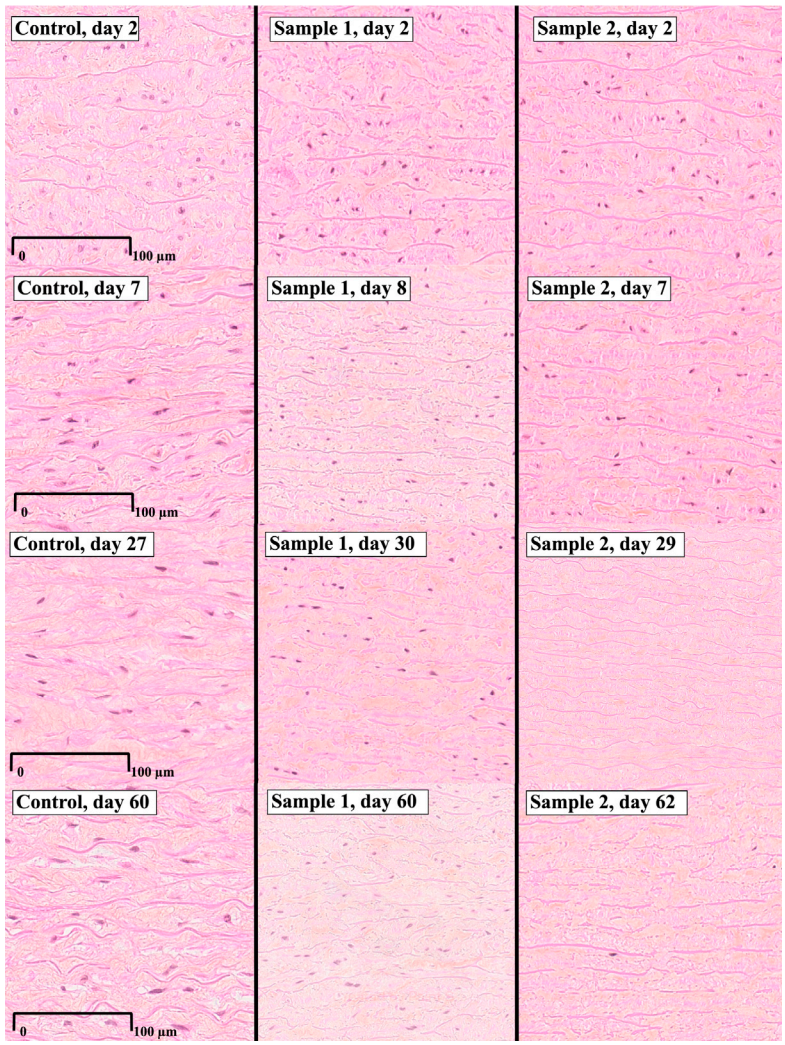


Figure 20. Cell appearance at different time points. Stained with erythrosine saffron, 20x magnification.

There were no differences when comparing elastic fibers at different times (Figure 21).

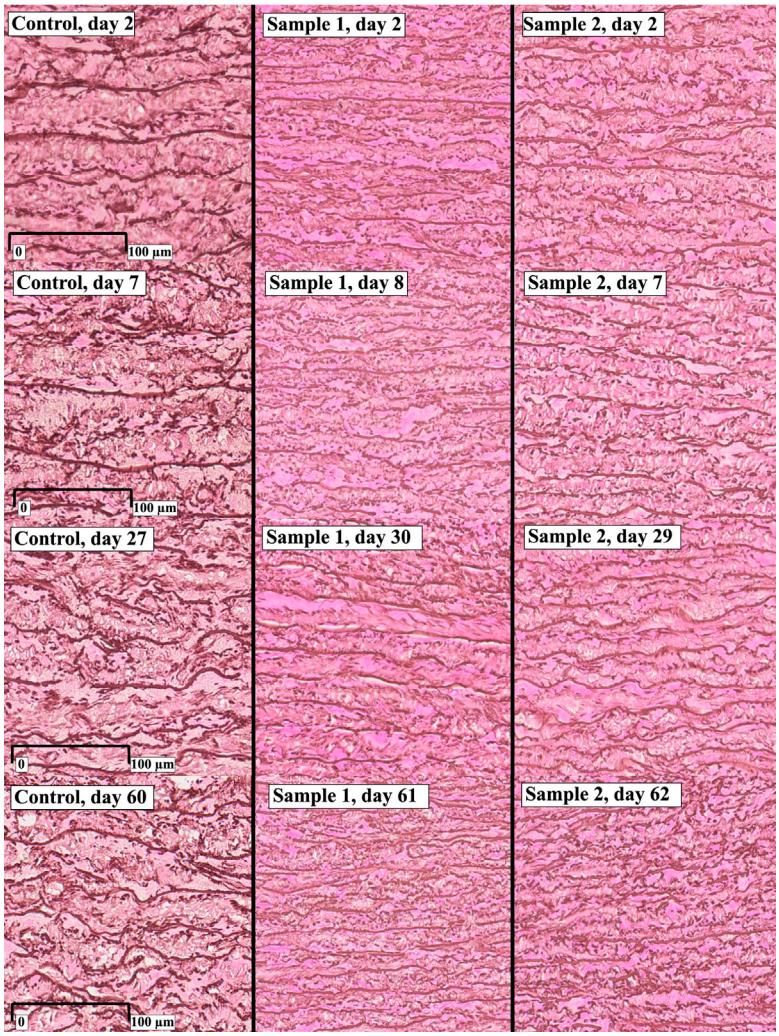


Figure 21. Elastin appearance at different time points. Stained with elastica van Gieson, 20x magnification.

There were no differences when comparing collagen fibers at different times. However, homografts that had undergone cryopreservation and mechanical testing (Samples 1 and 2) seemed to have less structured collagen fibers compared to the control sample that had not been cryopreserved nor mechanically tested (Figure 22).

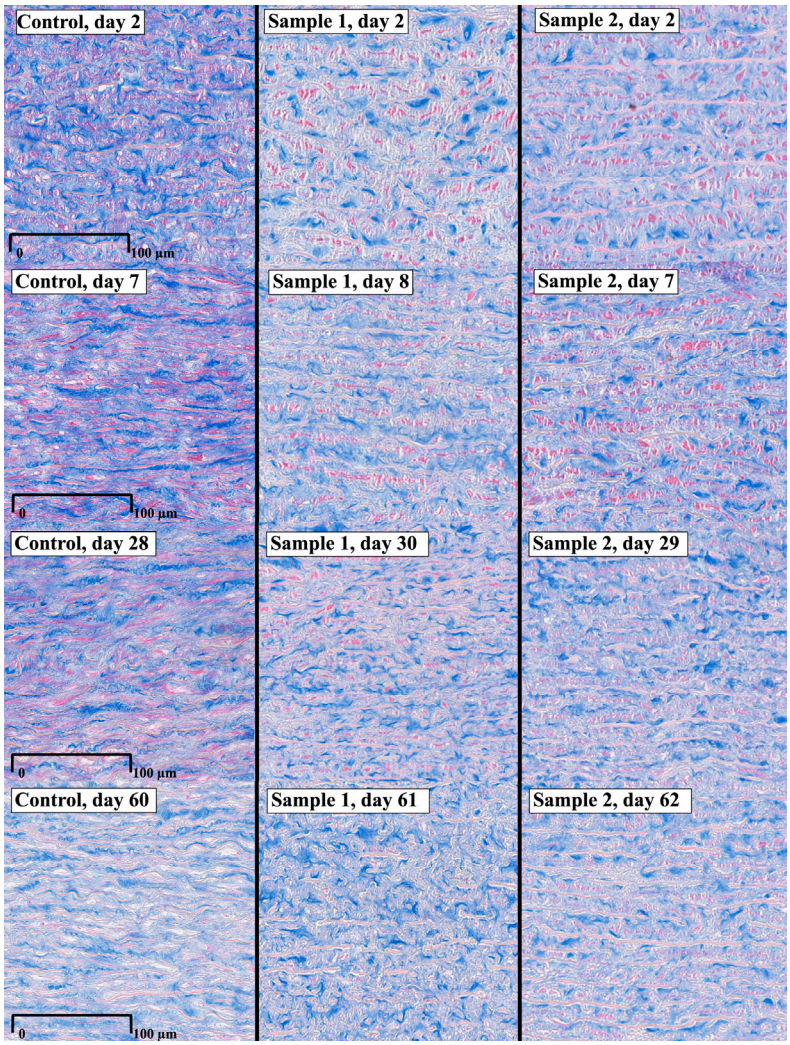


Figure 22. Collagen appearance at different time points. Stained with azan, 20x magnification.

Study V

Donors and homografts

Donor and homograft characteristics are presented in Table 25. All donors were MOD, hence there was no ischemic time prior to heart explantation. Heart collection to preparation time is defined as the time from heart retrieval until the homografts are being prepared at the tissue bank. During this time, the heart was stored in a cold environment in Ringer-acetate without antibiotic treatment. Only one aortic and one pulmonary homograft had a collection to preparation time that exceeded 48 hours.

Table 25. Donor and homograft characteristics.

Variable	Aortic homografts (n = 20)	Pulmonary homografts (n = 12)
Donor age, median (range)	53 (24-65)	51.5 (40-64)
Donor gender		
Female	9 (45%)	8 (67%)
Male	11 (55%)	4 (33%)
Discard reason		
Fenestration		11 (92%)
Atheromatosis	9 (45%)	
Valve leakage	7 (35%)	
Preparation damage	2 (10%)	
Unknown	1 (5%)	
Suspected cusp vegetation	1 (5%)	1 (8.3%)
Heart collection to preparation, hours (median, range)	36.5 (5-58)	35 (10-58)

Morphological interpretation

Each category was analyzed nine different times (each time point compared separately to the reference point), resulting in a Bonferroni corrected value of 0.0056.

Light microscopy

Each homograft (n = 32) generated biopsies from 10 different times. Samples were missing from day 0 from two aortic homografts and day 60 from one pulmonary homograft, resulting in 317 samples that were included in the analysis. Of 317 samples, 60 (19%) were randomly selected for blinded evaluation by a second evaluator to assess the validity of the protocol. The results from the inter-rater reliability analysis are presented in Table 26. The results vary from 75 to 95% agreement, which is considered substantial to almost perfect according to Landis and Koch (130).

Table 26. Inter-rater reliability calculated with intraclass correlation for continuous variables and weighted Cohen's Kappa agreement for categorical variables. CI = confidence interval.

Variable	Intraclass correlation	Cohen's Kappa agreement	CI (95%)
Cell count	95%		91-97%
Cell Appearance		75%	68-83%
Elastin Appearance	89%		82-93%
Collagen Appearance		86%	78-93%

The result of LM evaluation is presented in Table 27 for aortic homografts and Table 28 for pulmonary homografts. Only cell count in aortic homografts showed a significant difference compared to day 0.

Transmission electron microscopy

Samples were missing from day 0 from 11 aortic and seven pulmonary homografts. Samples from day 1 were missing from two aortic and one pulmonary homograft. Samples from day 21 were missing from one aortic and one pulmonary homograft. Samples from day 60 were missing from one pulmonary homograft. In total, 296 samples were included in the analysis.

TEM evaluation results are presented in Table 29 for aortic and Table 30 for pulmonary homografts. Cell count was significantly decreased from day 3 and beyond in aortic and day 4 and beyond in pulmonary homografts compared to day 1. Elastin appearance was significantly more degenerated at day 60 for aortic and day 21 (only) for pulmonary homografts. Collagen appearance shows significantly more degeneration at day 28 (only) for aortic homografts. There were no significant differences in collagen appearance for pulmonary homografts.

Table 27. Result from light microscopy evaluation of aortic homografts.
Day 0 is used as a reference point. Relative differences are calculated with the Wilcoxon signed rank test. *Significant with Bonferroni corrected p-value (<0.0056).

	Cell count			Cell appearance			Elastin appearance			Collagen appearance		
Day	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
0	1.0			1.0			1.0			1.0		
1	0.91	0.68-1.4	0.90	1.0	0.66-1.5	0.40	1.0	0.71-1.9	0.49	1.0	0.82-1.2	0.59
2	1.03	0.69-1.2	0.77	1.0	0.67-1.3	0.81	0.62	0.37-2.0	0.96	1.0	0.73-1.1	0.38
3	0.85	0.70-1.1	0.31	0.79	0.63-1.3	0.18	1.4	0.76-3.0	0.059	1.0	0.75-1.3	0.57
4	0.90	0.66-1.2	0.33	0.94	0.67-1.3	0.51	0.61	0.17-1.7	0.65	1.1	0.83-1.5	0.18
7	0.95	0.82-1.2	0.61	0.85	0.67-1.1	0.29	1.1	0.47-1.8	0.50	1.0	0.73-1.2	0.65
14	0.91	0.64-1.6	0.48	0.92	0.56-1.4	0.69	0.64	0.38-1.2	0.32	0.94	0.66-1.5	0.88
21	0.98	0.57-1.3	0.91	0.75	0.6-1.3	0.24	0.56	0.38-1.2	0.22	1.0	0.64-1.2	0.40
28	0.90	0.54-1.3	0.32	0.67	0.5-1.2	0.12	1.1	0.20-2.1	0.36	1.0	0.80-1.3	0.97
60	0.57	0.51-0.97	0.0019*	0.80	0.50-1.0	0.0070	0.75	0.38-1.5	0.72	0.93	0.80-1.2	0.71

Table 28 Result from light microscopy evaluation of pulmonary homografts.
Day 0 is used as a reference point. Relative differences are calculated with the Wilcoxon signed rank test.

	Cell count			Cell appearance			Elastin appearance			Collagen appearance		
Day	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
0	1.0			1.0			1.0			1.0		
1	0.79	0.63-1.0	0.041	1.0	1.0-1.3	0.083	0.86	0.52-1.5	0.81	1.2	1.0-1.7	0.049
2	0.80	0.60-0.91	0.10	1.1	0.90-1.3	0.23	1.2	0.83-2.3	0.22	1.2	0.92-1.6	0.064
3	0.88	0.49-1.0	0.071	1.2	0.90-1.3	0.15	1.0	0.83-1.7	0.41	1.3	1.0-1.75	0.022
4	0.92	0.75.1.1	0.48	1.1	1.0-1.5	0.096	1.0	0.48-1.3	0.75	1.0	0.57-1.75	0.61
7	0.83	0.52-1.03	0.071	1.0	0.93-1.1	0.65	1.1	0.64-2.7	0.25	1.1	1.0-1.5	0.077
14	0.74	0.63-1.5	0.91	0.89	0.83-1.1	0.45	1.1	0.50-1.5	0.61	1.0	0.78-1.1	0.52
21	0.88	0.61-1.5	0.94	0.93	0.73-1.1	0.69	1.0	0.65-1.2	0.75	1.0	0.93-1.3	0.49
28	0.97	0.57-1.3	0.70	0.65	0.53-1.0	0.090	1.1	0.75-2.1	0.15	1.2	0.92-2.0	0.17
60	0.67	0.51-1.2	0.21	0.60	0.50-0.78	0.029	0.92	0.43-1.3	0.56	1.3	1.0-1.5	0.045

Table 29. Result from transmission electron microscopy evaluation of aortic homografts.
Day 1 is used as a reference point. Relative differences are calculated with the Wilcoxon signed rank test. *Significant with Bonferroni corrected p-value (<0.0056).

	Cell appearance			Elastin appearance			Collagen appearance		
Day	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
0	1.0	0.98-1.1	0.44	0.95	0.95-1.2	1.0	1.0	1.0-1.1	0.34
1	1.0			1.0			1.0		
2	0.95	0.89-1.0	0.013	0.95	0.92-1.9	0.14	1.0	0.89-1.1	0.78
3	0.88	0.81-0.92	<0.001*	1.0	0.90-1.1	0.75	1.0	0.91-1.1	0.99
4	0.84	0.77-0.93	<0.001*	0.95	0.90-1.1	0.20	1.0	0.95-1.1	0.82
7	0.82	0.77-0.89	<0.001*	0.97	0.90-1.0	0.30	0.96	0.80-1.1	0.38
14	0.67	0.62-0.78	<0.001*	0.90	0.83-1.0	0.025	0.98	0.84-1.1	0.47
21	0.67	0.63-0.76	<0.001*	0.92	0.80-1.0	0.073	0.93	0.85-1.0	0.23
28	0.64	0.55-0.68	<0.001*	0.90	0.82-0.99	0.014	0.86	0.80-0.93	0.0013*
60	0.53	0.48-0.58	<0.001*	0.90	0.83-0.99	0.0051*	0.95	0.90-1.1	0.57

Table 30. Result from transmission electron microscopy evaluation of pulmonary homografts.
Day 1 is used as a reference point. Relative differences are calculated with the Wilcoxon signed rank test. *Significant with Bonferroni corrected p-value (<0.0056).

	Cell appearance			Elastin appearance			Collagen appearance		
Day	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*	*Median*	*IQR*	*p-value*
0	1.1	1.0-1.2	0.14	1.0	0.89-1.1	0.89	1.0	0.96-1.2	0.59
1	1.0			1.0			1.0		
2	0.98	0.94-1.0	0.46	1.0	0.94-1.1	0.61	1.0	0.87-1.1	0.91
3	0.93	0.87-0.99	0.041	0.97	0.93-1.0	0.31	1.0	0.93-1.2	0.35
4	0.87	0.78-0.94	0.0037*	0.97	0.88-1.0	0.084	1.1	0.89-1.1	0.94
7	0.78	0.76-0.86	0.0022*	0.89	0.85-1.0	0.028	1.0	0.76-1.1	0.31
14	0.78	0.69-0.93	0.0022*	0.99	0.91-1.1	0.78	0.90	0.81-0.97	0.054
21	0.68	0.53-0.73	0.0033*	0.91	0.82-0.97	0.0049*	1.0	0.84-1.1	0.86
28	0.71	0.67-0.79	0.0022*	0.97	0.94-1.0	0.41	1.0	0.81-1.1	0.97
60	0.55	0.49-0.71	0.0033*	0.99	0.91-1.0	0.31	0.91	0.84-0.97	0.11

Discussion

Main findings

Study I	Young donor age was a significant risk factor for early reintervention. A homograft ischemic time of 1-24 hours led to a higher reintervention rate in homograft recipients compared to 0 hours. However, >24 hours did not have a higher reintervention rate. When comparing 1-24 hours and >24 hours separately, there was no difference in reintervention rate.
Study II	Structural defects of the homografts are the major reason for discard during processing at the tissue bank in Lund. Minor structural changes, such as small fenestration or minor atheromatosis, did not lead to a higher reintervention rate in recipients compared to homografts without minor structural changes.
Study III	The contamination rate of homografts was 31% prior to decontamination, but most were treated successfully with antibiotic decontamination. Contamination prior to antibiotic decontamination did not lead to a higher reintervention rate nor a higher risk for endocarditis compared to homografts that were never contaminated.
Study IV	Elastic modulus decreased significantly when prolonging antibiotic decontamination to >7 days prior to cryopreservation. Yield stress and energy at yield stress varied at different times.
Study V	Cell count decreased in aortic homografts investigated with LM after 60 days of decontamination. It was easier to detect differences in TEM where deterioration of cell appearance could be seen at three and four days and beyond for aortic and pulmonary homografts respectively. Deterioration of elastin appearance could be seen at day 60 for aortic and day 21 for pulmonary homografts respectively. Deterioration of collagen appearance could be seen at day 28 for aortic homografts.

Interpretations and implications

Homografts are among the best conduits to use for RVOT reconstruction. Since its introduction as a RVOT conduit in 1966, the homograft has been an essential part of surgical corrections of congenital heart diseases (9). In 1987, accessibility increased even further when cryopreservation was introduced as a storage method, prolonging the possible storage times from a few months to several years (81). However, the homograft is a biological valve, and it has limited durability. In our material, freedom from reintervention was 76% at five years and 57% at 20 years which are excellent results, especially when considering that we investigated relatively young patients (median age 9.9 years) with only a small proportion of Ross procedures (16% of the whole group). Still, most patients require multiple reinterventions during a lifetime, and it is important for tissue banks and surgeons to strive for maximal homograft durability.

For surgeons

There are some risk factors for early reintervention that surgeons can consider. **Study I** identify the use of aortic homografts and young donor age as risk factors for reinterventions. In repeated studies, aortic homografts have shown inferior

durability and earlier development of stenosis and calcifications compared to pulmonary homografts (13,18,23–25,30,34–36). When possible, a pulmonary homograft should be the first-hand choice when considering a homograft for RVOT reconstruction. Studies even suggest that the Contegra conduit could be a better alternative if only aortic homografts are available (53). However, there is a particular subgroup of patients where the homograft type seems to have less importance. Very young patients (<1 years) have a high risk of developing early conduit dysfunction, not because of outgrowth but because of an accelerated calcification of the homografts compared to older patient groups (42). Among these patients, homograft durability is short in all types of conduits, and the outcomes of aortic and pulmonary homografts are comparable (31,109).

Considering donor age, our results in **study I** show that young donor age is a risk factor for early reintervention, even when excluding small-sized homografts (<18 mm) from the analysis. This result indicates that young donor age could be an individual risk factor and not only influenced by the correlation with smaller homograft sizes. Kalfa et al. and Gerestein et al. could show better performance of homografts from donors >30 years compared to younger donors, which could be due to stronger viability in young donors (13,36). Increasing donor age has a negative correlation with the percentage of viable cells in the homografts (110) and considering that the immune response might play a role in homograft degeneration, this could be an explanation for our and others' results. In conclusion, further studies are necessary to investigate the exact role of donor age in homograft durability, but surgeons do not need to worry that choosing a homograft from an older donor would provide tissue of less quality. Today, most tissue banks have an age limit of 60-65 years for homografts, but studies suggest that older donors (up to 80 years) could provide pulmonary homografts with good tissue quality (32,79).

For tissue banks

When developing protocols for homograft procurement, preparation, decontamination, cryopreservation, and quality control, tissue banks in Europe use guidelines in combination with local experience and opinion. The lack of strictly scientific evidence for many guidelines leads to a high influence of local expertise, and a plethora of different protocols and routines at different tissue banks (19,79). Consequences are a high variability in the rate of discarded homografts (19-65%) and difficulty in comparing outcomes from different tissue banks. But on the aspects of ischemic time and microbiological contamination, tissue banks should be able to agree.

Ischemic time

Studies I, IV and V investigate the importance of ischemic time in different steps of the homograft collection process.

Study I compare outcomes in recipients of homografts with different ischemic times prior to explantation (0, 1-24 or >24 hours). The results show the lowest reintervention rate in recipients with 0 hours of ischemic time, but homografts with >24 hours of ischemic time had a lower reintervention rate compared to 1-24 hours. At the tissue bank in Lund, the maximum ischemic time prior to explantation is 48 hours, with a maximum of six hours of warm ischemic time. According to guidelines, the maximum ischemic time should be 24 hours, which most tissue banks adhere to (19,79). Seeing that results after homograft implantation improved after the introduction of cryopreservation compared to prolonged storage at 4°C for up to 8-12 weeks, it seems that the homograft loses its quality at some point at prolonged ischemic times (81,111) but we do not know when this deterioration starts. According to our results, there is no harm in extending the cold ischemic time to 48 hours. Our results are comparable to other studies investigating different ischemic times in relation to recipient outcome, also showing no differences in outcome when prolonging the maximum ischemic time to 48 hours (17,36). Smit et al. and Bester et al. have also looked more closely at the homograft tissue at prolonged ischemic times and have not been able to show any negative effects on the tissue when expanding the maximum ischemic time to 48-72 hours in sheep. They investigated the tissue both directly after harvest and after 180 days of implantation in a sheep model and found no differences in mechanical properties, extracellular matrix structure, echocardiographic examination of the implanted homografts and immunological markers after explantation between different time intervals (112,113).

When limiting the maximum ischemic time, the proportion of possible donors decreases (33). In our material, 41% of the implanted homografts had an ischemic time of >24 hours.

Study IV is the first to evaluate the mechanical properties of the homograft in relation to prolonged cold ischemic time during antibiotic decontamination, but similar measurements have been made to investigate other aspects of the homograft process. As mentioned, Smit et al. and Bester et al. show no differences in mechanical properties when prolonging the cold ischemic time prior to heart explantation (112,113). In addition, Fiala et al. investigated mechanical properties after prolonged cryopreservation times and showed no differences when prolonging the cryopreservation for up to 10 years (93). Both studies show similar values of elastic modulus and yield stress compared to our study. Our results show that both elastic modulus and yield stress were highest at the shortest decontamination time that was investigated (two to four days) and lowest after seven to nine days. The decrease in mechanical properties could indicate a weakening of either elastic fibers or collagen.

Study V further investigates the presence of autolytic changes and signs of degeneration in homografts with prolonged cold ischemic time during antibiotic decontamination. The results confirm that cellular degeneration occurs early, but

there were no significant differences in elastic fiber and collagen structures in either LM or TEM until day 21 and day 28 respectively.

Evaluation with LM could only show that cell count decreased significantly in aortic homografts after 60 days of decontamination. No other differences were detected. LM could only study the tissue at 40x of magnification, giving a good view of the overall structural integrity but without the possibility to study the details of cells and fibers. At this magnification, the structural integrity of the tissue seems to be well preserved for up to 60 days of decontamination.

Evaluation with TEM gave more detail, showing degeneration of cell nuclei after three to four days of antibiotic decontamination with increased condensation of chromatin, dissolution of the cell nuclei membrane and detachment from surrounding tissue. It is shown that cellular viability decreases with increased ischemic time, both warm and cold (80,96,110). Arguments that advocate the importance of viable cells say that surviving cells could maintain the extracellular matrix and keep the endothelial surface intact (82). Studies have shown the presence of donor cells after explantation of homografts (at autopsy or due to surgical reintervention), but it has not been possible to determine their exact function and importance for homograft durability (45,82,114–116). Even if viable donor cells can be detected several years after explantation, the extracellular matrix still shows degeneration with loss of structural integrity and architecture (45,114,117). The presence of donor cells could also elicit a stronger immune response in the recipient, which some argue could lead to faster degeneration (118,119). It has been proved that donor-specific antibodies and helper- and cytotoxic T-cells arise in the blood of the recipient after homograft implantation. Their clinical importance has not been demonstrated, but the pattern of immune reaction is comparable to that in rejection after heart transplantation (119–122). However, when examining explanted homografts, cusps and vessel wall only show occasional infiltration of inflammatory cells in adult patients, suggesting that no major immune response has occurred (40,45,117). When looking at the pediatric population, results are conflicting, where some studies have found large infiltration of inflammatory cells after explantation (40,41), while other studies could not show any differences from the adult patients (45,117). In recent years, the use of DHs shows promising results which indicates that the recipient does not depend on viable cells from the donor to ensure homograft function (66,73). To date, there is no clear consensus on whether cell viability is crucial for homograft durability, but the good performance of DHs indicates that it is not necessary to strive for viable cells prior to cryopreservation.

Except for remaining donor cells, studies have shown that the recipient will repopulate the homografts with their own cells after implantation, but it has not been shown that these cells would have a normal function either (114,123,124).

The impact of prolonged ischemic time on extracellular components has not been studied to the same extent as cells. Livi et al. investigated 20 homografts (10 aortic and 10 pulmonary) after two and four weeks of cold ischemic time in antibiotic decontamination and could not see any differences in LM compared to the day of

harvest, either in the elastic tissue nor the collagen structures (96). Fabian et al. investigated homografts in LM as well and showed that structural degeneration was correlated to higher donor age but was not affected by prolonged cold ischemic time prior to antibiotic decontamination (12-21 hours) or prolonged cold ischemic time during antibiotic decontamination (12-21 days) (125). The correlation between structural degeneration and increased age is well known from the literature (95). Studies of homografts investigated after explantation from recipients have shown that the donor collagen matrix can be largely intact several years after homograft explantation, suggesting that the structural basis of the homograft could be of greater importance than cell viability for the long-term performance of homografts (40,115). The extracellular matrix is better kept in homografts that are explanted from patients for technical reasons (non-cardiac death or external compression of the valve) than in homografts that are explanted for degenerative changes, indicating that degenerating extracellular matrix contributes to homograft dysfunction (45). It is known from forensic studies that tissues with extracellular matrices that are rich in collagen and elastin, for example vessels, are more resistant to degeneration compared to parenchymatous organs, and that the overall autolytic process slows down significantly if the tissues are stored in a cold environment (126–128).

The results in **study IV** and **study V** do not completely correspond. **Study V** could not demonstrate any differences in the extracellular matrix that could explain decreasing values of elastic modulus and yield stress at only seven days of ischemic time as shown in **study IV.** There is a possibility that visible morphological changes are delayed compared to mechanical property changes which might appear earlier. **Study V** includes more homografts with observations at closer time intervals compared to **study IV.** As mentioned in the limitations, there were some difficulties with the measurement set-up in **study IV** that could affect the outcome.

Macroscopic control

Study II investigated the impact of small fenestrations and minimal atheromatosis in the homograft and its impact on the long-term outcome of the homograft in the recipient. The results do not show any differences in outcome between homografts with minimal structural impairment and homografts without any structural impairment.

Structural impairment is the main reason for discard at the tissue bank in Lund. Of all incoming homografts, 45% are discarded, and of all discards, 67% are due to structural impairment. Other tissue banks describe that 42-58% of their discards are due to structural impairment (87,91,129). The lower proportion at other tissue banks could be due to less strict criteria, other inspection methods, or that the tissue bank in Lund has a different practice on discard due to microbiological decontamination compared to other tissue banks, making our proportion of discards due to contamination very low compared to other banks (79). Our results show that our protocol, derived from the guidelines, leads to a high proportion of homograft discards, but that the impairments that are accepted are safe. However, the

recommendations are strict, and only 8.0% of the implanted homografts had any kind of remarks. In the literature, there are no other studies investigating small impairments of the homografts and their impact on outcomes after implantation. If such studies were made, showing similar results to ours, one could discuss whether it is possible to be more liberal with the protocols. But up to this date, our data is too small (only 38 patients receiving a structurally impaired homograft for 24 years) to suggest any changes in guidelines or protocols.

Microbiological burden

Study III could not demonstrate any impact of microbiological contamination during homograft procurement on long-term outcomes or the prevalence of endocarditis in recipients. In our material, the contamination rate was higher in NHBD (44%) compared to MOD (20%) and domino donors (17%) but there were no differences in contamination rate in NHBD with a maximum of 24 hours of ischemic time compared to >24 hours. Other tissue banks have described a contamination rate of 33-58% in NHBD with a maximum of 24 hours of ischemic time, which is comparable to our results even though we used a prolonged ischemic time (85,86,130).

Three homografts had positive tissue cultures at implantation, most likely from contaminations that occurred during unpacking. All recipients recovered well, with no signs of endocarditis during follow-up. Jashari et al. had a similar experience from their tissue bank, describing three cases with positive cultures at implantation (aspergillus, multi-resistant *Staphylococcus aureus,* and *Enterobacter cloacae).* These patients recovered well, and they also assessed the positive findings as contaminations in the operating room (91).

In our material there were 18 cases of endocarditis during follow-up, with no difference in prevalence between recipients of homografts that were contaminated or not prior to antibiotic decontamination. There were two cases of endocarditis that had the same species of microbe in blood cultures at endocarditis diagnosis as the homograft had prior to antibiotic decontamination (alpha-hemolytic *streptococci*). Considering the disease-free interval (19 years and two years respectively) and that the microbe is sensitive to antibiotics used in the antibiotic cocktail at the tissue bank in Lund, transmission from the homografts seems unlikely.

There are few reports in the literature on the possible transmission of microbes and fungi from donated cardiovascular tissue to a recipient. Only one case report clearly describes such a transmission. In 1996, one recipient operated in the USA received a homograft in the aortic position that was contaminated with *Candida albicans* prior to decontamination, where follow-up cultures had been negative. Sixteen days after surgery, he developed endocarditis from *Candida albicans*. The homograft had to be explanted and replaced, and genetic analysis showed that the *Candida albicans* that was found at explantation had high genetic similarities to the *Candida albicans* found at homograft procurement (prior to decontamination), but sensitivity to fluconazole and amphotericin B had decreased in the isolates from the

explanted homograft (131). Wang et al. summarizes all reported homograft infections in the USA during 2001-2004, showing 35 reported cases with nine deaths. There are no more details on the cases, and both Wang et al. and reports from others describe the difficulties in differentiating homograft and other allograft transmissions from postoperative infections due to other causes (132,133). The World Health Organization provide a notify library where all serious adverse events in medical products of human origin should be reported. In this library, there were seven reported cases of possible microbiological or fungal transmission from homografts to recipients, summarized in Table 31 (134). There is no information on the year in which the cases were reported. According to the EDQM, all serious adverse events should be reported through this system, but the actual adherence to this system is unknown.

Table 31. Summary of all serious adverse events reported to the notify library on possible microbiological transmissions from homografts to recipients.

	Microbe	Time interval	Description
Case 1	*Candida albicans* (fungi)	5 months	Homograft in aortic position. Development of endocarditis with severe complications. DNA testing proved it was the same organism as found during processing.
Case 2	*Oerskovia Tubarata*	6 months	The register does not define what type of infection the microbe caused but describe that an infection was present and that the same organism was present in the donor at tissue processing.
Case 3	*Arthtographis kalrae* (fungi)		Time interval not defined. Recipient died from infection after 13 months. Speculates that it was donor-derived but not proven.
Case 4	*Paecilomyces variotii* (fungi)	8 months	Homograft in aortic position. No actual suspicion that the infection was derived from the homograft but cannot be ruled out.
Case 5	*Mycobacterium tuberculosis*	8 months	Few details, recipient presented with pneumonia and heart murmur. Homograft does not seem to have been tested for mycobacterium prior to implantation but transmission cannot be ruled out.
Case 6	*Prevotella oralis*	19 days	Homograft in aortic position. Possible endocarditis, positive blood cultures. Homograft was excised but without macroscopic signs of infection. Gram-staining of tissue after explantation showed gram-negative rods but no cultures were positive. No information about the donor and contamination during processing.
Case 7	*Cardiobacterium hominis*	2 years	Homograft in aortic position. Definite endocarditis, surgical intervention with heart valve replacement. No information on donor and contamination during process, but very low suspicion of homograft origin due to the long-term interval.

The results from this search also show that it can be difficult to determine the origin of an infection. At least two of the seven cases (cases 1 and 2) seem to have originated from the donor. In case 3, the suspicions are strong, but the background of the suspicions are not described. The rest of the cases suggest a possible but not

likely transmission from the homograft. An interesting observation is that all cases where the homograft position is defined show endocarditis in the AV position.

The results from the univariable Cox proportional hazard regression show a significantly higher reintervention rate in homografts contaminated with high-risk microbes compared to no contamination, but this result is not reproducible in the multivariable model. The result in the univariable analysis is probably due to the overrepresentation of known risk factors in the high-risk contamination group, with lower recipient age and a higher proportion of extra-anatomical implantations compared to the other groups (Table 19).

Criteria for discarding tissue due to microbiological contamination need to be discussed. When strictly following guidelines, a high proportion of homografts will be discarded. These guidelines are mainly derived from opinions and experiences, since references only refer to the activity of different antibiotic cocktails with no investigation of outcome in recipients (135). In our material there were no cases of homograft-transmitted endocarditis and no increased risk of early homograft reintervention due to contamination during processing. According to other available data, the transmission of microbes from homograft to recipient is very rare. Of the few reports available, fungal infections seem to have caused the most serious infections (case report from the USA and cases 1 and 3 in Table 31).

Suspected homograft-transmitted infections must be reported and documented, and thorough evaluation of homografts prior to acceptance for transplantation must be conducted.

Strengths and limitations

Strengths

In **studies I-III**, there was a minimal loss to follow-up (1.0-1.6%). In Sweden, surgical RVOT reconstructions with homografts and other conduits are only conducted at two hospitals (Skane University Hospital and Sahlgrenska University Hospital), meaning that the recipients from our center well represent the patient group that needs RVOT reconstructions in Sweden. Sweden also has a national register for all ACHD (Swedcon), that is routinely updated after patients revisit at the local cardiologist, making the follow-up almost 100% complete.

Limitations

In **studies I-III,** all implanted homografts are included as one group. This group has a high heterogenicity according to age, severity of diagnoses and past medical history. This could make the results hard to generalize to groups of patients with specific characteristics, such as very young patients or patients with high complexity heart defects.

In **study II**, there are suspicions about missing data on homografts that would lead to higher discard rates than reported. Looking at time periods, the proportion of discards due to structural impairment was less frequent before 2005 (data not shown). In recent years, the importance of strict data registration in tissue banks has been noticed, and major efforts have been made to improve the registration in Lund. Structural impairment was probably not absent in the early years, but homografts that were discarded were probably not registered at all. In our material, 18% of the homografts were collected prior to 2005.

In **study III**, there is a risk of false negative culture results, especially after antibiotic decontamination where residual antibiotics could inhibit culture growth (136,137). The sensitivity of our methods in comparison to other tissue banks has not been evaluated, decreasing the generalizability of the results regarding the proportion of contamination.

When performing mechanical testing in **study IV,** all samples were mounted tight to the grips to avoid slipping. As a result, most samples ruptured close to the grips, raising the concern that the grips may have damaged the tissue locally. If local damage occurred, it would not affect the elastic modulus but could give lower values for yield stress and energy at yield stress. All samples were mounted in the same way, but the group of two to four days had longer and wider samples compared to the other groups, making them easier to mount. Both **study IV** and **study V** include small groups of homografts (10 and 32 respectively). For three years, all eligible tissue was collected, and the groups could not be expanded further without significantly increasing the study time.

Conclusions

Study I

Young donor age is a risk factor for early reintervention. Homografts from older donors had superior long-term outcomes, even when corrected for homograft size in a subgroup analysis. The cold ischemic time prior to heart collection can be expanded to 48 hours without affecting the long-term outcome in recipients. Many tissue banks struggle with a lack of suitable donors and a prolonged maximum cold ischemic time from 24 hours to 48 hours would increase the possible donor pool.

Study II

Small fenestrations of the homograft cusps and minor atheromatosis of the vessel wall do not impact the long-term outcome after RVOT reconstruction. Current guidelines on structural impairment seem acceptable considering homograft quality.

Study III

Contamination of homografts prior to decontamination does not affect the long-term outcome of homograft performance after implantation. Transmission of microbes during homograft implantation seems to be rare, but over- and under-reporting may be present due to diagnostic difficulties. Many tissue banks report contamination as their main reason for homograft discard during processing. Our results suggest that there is no harm in using homografts that are positive prior to decontamination if follow-up cultures after decontamination are negative.

Study IV

The highest values of elastic modulus, yield stress and energy at yield stress were found at two to four days of decontamination. Values decreased at seven to nine days and beyond for elastic modulus and seven to nine days and 28-30 days (but not 60-62 days) for yield stress. No significant differences were found for energy at yield stress. The results could indicate that some deterioration of elastic fibers and/or collagen occurs with a prolonged decontamination time, resulting in reduced stiffness and resistance to maximal tensile stress. The clinical significance of these results remains to be clarified.

Study V

When studying signs of degeneration in homografts prior to implantation, detailed investigation of tissue is necessary. The overall structural integrity as seen in LM was kept for at least 60 days, where the only difference that could be found was a decrease in cell count at 60 days in aortic homografts. When using TEM, details of individual cells and fibers could be studied, and evidence of cell degeneration could be found after a total of three or four days of decontamination, preceded by a median cold ischemic time of 35 and 37 hours prior to homograft preparation in aortic and pulmonary homografts respectively. Earliest signs of elastin and collagen degeneration could be found after 21 and 28 days respectively, showing high resistance to degenerative processes. We suggest that homograft decontamination in a cold environment could be prolonged to at least 14 days without affecting the quality of the tissue matrix.

Future directions

The homograft is a well-proven conduit for RVOT reconstruction, but limited durability and availability continue to be its main disadvantages. It is difficult to determine how to improve the durability even further since all biological heart valves show some degree of degeneration and stenosis development over time, with the homografts being one of the heart valves with the greatest durability. The exact mechanism behind homograft degeneration needs to be further investigated. Some suggest that an immune response might accelerate homograft degeneration, with studies showing increased levels of antibodies and immunoreactive cells after implantation. Based on these findings, decellularization of homografts was introduced and has shown promising results in terms of long-term durability. However, the decellularization of homografts is still a complicated and expensive process, with costs that extend to up to three times that of a standard homograft (71). Therefore, definite superiority remains to be proven before DHs should be used as the standard conduit.

The second main disadvantage of homografts is their limited availability. It is difficult to determine precisely how significant this problem is. To our knowledge, there are no registers showing how often a surgeon would prefer a homograft but is forced to choose another conduit, nor how often a surgeon needs to deviate from the original plan and choose a homograft of a different type or a different size due to a lack of available homografts. If introducing such registers at departments for cardiothoracic surgery, it would be easier to trace how large this problem is.

Strict guidelines have significantly decreased the amount of available homografts. Of all homografts that are acquired at tissue banks, up to 50% could be discarded due to different contraindications of the tissue, such as contamination, damage or structural impairment (79). In addition to the homografts that are discarded in the tissue banks, many donors are turned down before even explanting the heart, due to contraindications such as age or prolonged ischemic time. Our results suggest that some of these discards could be avoided without compromising homograft quality, but there are few similar studies and the reproducibility of our results needs to be proved. In addition to donor characteristics, structural impairment, microbiological contamination, and ischemic time that together represented the focus of our studies, other guidelines should be evaluated as well. For example, donor age limits are an interesting area, since pulmonary arteries and valves often show good macroscopic quality even at increased ages (32).

”Success in medicine, as in any field, requires not only technical competence, but also the ability to communicate effectively and work collaboratively”

Atul Gawande

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