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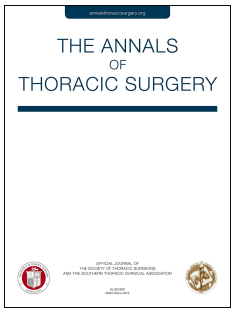


Early Insight into In-vivo recellularization of cell-free allogenic heart valves

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**Early Insight into In-vivo recellularization of cell-free allogenic heart valves**

Running Head: Repopulation of cell-free homografts

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**Abstract**

***Background.*** Unlike the vast amount of animal data available on the recellularization of allogenic decellularized heart valves (DHV), there have only been sporadic histological reports on such grafts in humans.

***Methods.*** Two experienced cardiac pathologists independently evaluated human specimens obtained during re-operation between 12/2010 and 4/2017 DHV in 7 categories following automated staining (scores 0-6) in comparison to published data. An optimal result of 42 points was classified as 100%.

***Results***. 364 DHV, 236 pulmonary (DPH) and 128 aortic (DAH) were implanted, freedom from explantation was 96.1% (DAH) and 98.7% (DPH). Re-operations were due to (suspected) endocarditis in 5/11, stenosis either at subvalvular/valvular /supravalvular level in 3/11, planned staged re-operation in 2/11 and 1 heart transplant. Good reader agreement was reflected by an inter-agreement weighted *K*appa of 0.783 (0.707-0.859, 95% CI).

The relative histological score in non-endocarditis cases was 76% (±4.3, max.81%). Intracellular pro-collagen type 1 production was found in recipient mesenchymal cells within the transplanted grafts. In endocarditis cases the histological score was significantly lower with 48% (±7.3, min.41%, p=0.0004) due to leucocyte infiltration and matrix degradation. 1 DPH showed immune system mediated graft failure. Grafts obtained during the first 12 months after implantation were not evenly repopulated with less recellularization in the inner parts; no difference was found between DAH and DPH with respect to extent of recellularisation.

***Conclusions***. Significant in-vivo recellularization with non-inflammatory cells was observed in this study. Spontaneous recellularization appears to require multiple months, which correspondingly has an impact on size selection for growing patients.

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*Supplemental Tables: 3*

**Key words:** Heart valve disease; tissue engineering; decellularization; recellularization

Decellularized human heart valves (DHV) have been approved by multiple competent authorities for routine use. To date, several hundred DHV have been implanted for aortic and pulmonary valve replacement and have yielded promising mid-term results.[1-5]

Little, however, is known about the important process of DHV recellularization by the recipient`s cell populations. Even though a generally low level of immunogenicity has been demonstrated for DHV[6,7], infiltration by neutrophils, lymphocytes and macrophages may occur and can only be reliably assessed by histology. Furthermore, true long-term durability of DHV can only be expected if recellularization with endothelial cells, smooth muscle cells and fibroblasts occurs to an extent large enough to enable elastic fibers, collagen matrix structure, proteoglycans and other extracellular proteins to continuously regenerate.

In contrast to the vast amount of animal data available on the recellularization of decellularized allogenic heart valves, so far only sporadic histological reports on decellularized grafts have been put forward in humans.[8-11] Specifics of tissue processing, methods and results of histological analyzes albeit were heterogeneous, ranging from infiltration by inflammatory cells and severe calcification to good preservation of the matrix and widespread non-inflammatory repopulation.

This study aims to establish more histological evidence on the extent of spontaneous in-vivo recellularization of detergent based decellularized, non-cryopreserved and non-seeded allografts.

## Patients and Methods

The detergent based method used for decellularization of homografts has been described before.[1] After 2013 a spin-off company from Hannover Medical School provided the decellularisation service following market approval for DPH and DAH (PEI.G.11766.01.1 and PEI.G.11634.01.1). Completeness of decellularization was assessed via histology and DNA content for each homograft before release.

The postoperative clinical course of all patients receiving either a decellularized pulmonary homograft (DPH) for pulmonary valve replacement (PVR) or a decellularized aortic homograft (DAH) for aortic valve replacement (AVR) have been prospectively followed

since 1/2005. Approval was given by all local ethics committees before start of the studies as well as informed consent by all participants or parents.

All explanted homografts from 12/2010 to 4/2017 were secured for histo-pathological analysis. Specimens obtained during planned staged palliative operations were also harvested, when possible without performing any additional incision to the existing graft.

*Histological assessment*

Two experienced cardiac pathologists (> 8 years after board examination) independently evaluated the structure of the valve wall and cusp (when available) as well as the amount and type of recellularization, using a semi-quantitative score in integer numbers ranging from 0-6 points in relation to published normal histological findings.[12]

Formalin-fixed and paraffin-embedded tissue was cut to 1µm slices at a routine microtome. For standard and immuno-histochemistry the cut slices were stained at a routine automated tissue-stainer (Ventana Ultra, Roche, Basel, Switzerland) according the manufacturer's specification. Antibody details are provided as Supplemental Table 1. Standard histological analysis was performed using Haemotoxylin and Eosin (HE) and Elastica van Gieson (EvG) staining. Scores were allocated for the valves as follows: Destruction of the elastic fibers and collagen structure was graded with 0 points, poorly maintained matrix structure with 2 points, maintained appropriate matrix structure with 4 points and a normal or near-normal matrix structure was allocated 6 points. A normal or near-normal overall cell count within the homograft resulted in 6 points, moderate recellularization in 4 points, poor recellularization in 2 points and no recellularization in 0 points.

We also differentiated cell types within the explanted tissues by applying uniform automated immuno-histological staining to all specimens. Using the same scoring model we assessed endothelial cells, fibroblasts, myofibroblasts and smooth muscle cells as characterized by Vimetin. Cell counts for smooth muscle cells and myofibroblasts, as characterized by positive smooth muscle cell α-actin (SMA) was graded accordingly. Infiltration by granulocytes was characterized by CD 15 staining, T-lymphocytes by CD3 and B-lymphocytes by CD20 staining.

A total score was calculated for each patient using the results of 2 anatomical stainings (as not all specimens provided material for separate wall and cusp analysis) and 5 immunological stainings. An optimal result in these 7 categories would be calculated as a total of 42 points and classified as 100%. Overall relative results were calculated for each specimen using the mean overall point score of both observers.

*Statistical analysis*

Statistics on decellularized homografts were performed using SPSS 23 (IBM Corporation, Somer, NY). Summaries of the numeric data are given as means and standard deviation; a probability value of 0.05 or less was considered statistically significant. Inter-rater and intra-rater agreement (Kappa) was calculated with 95% confidence interval as reported by Fleiss. Kappa`s of 0.61-0.80 were considered good and 0.81-1.0 as very good.

No correlations between implant duration and recellularization rate were calculated due to the significant heterogeneity of the small study cohort with respect to age at implantation, reasons for re-operation and DHV position.

**Results**

A total of 364 decellularized, non-seeded and non-cryopreserved homografts (236 pulmonary and 128 aortic) were implanted between 1/2005 and 4/2017. Follow-up was 100%, comprising 888.1 patient years and >2700 examinations. Details on patient demographics, homograft size and overall hemodynamic performance are provided in Table 1.

Within this 12-year period 8 decellularized homografts were explanted, leading to freedom from explantation of 96.1% for DAH and 98.7% for DPH. One DAH was not available for standardized histological assessment; this case involving the rupture of the native ascending aorta due to an Aspergillus infection has been reported previously.[1]

Eleven specimens (7 explants, 4 biopsies) were available for histological assessment, 6 specimens derived from DAH, 5 from DPH. Details of specimens, patient characteristics and clinical situation are provided within Table 2.

*Reasons for re-operation*

Reasons for re-operation included scheduled re-operation in 2 out of 11 patients, stenosis either at subvalvular, valvular or supravalvular level in 3 patients and endocarditis/suspected endocarditis in 5 patients. Intraoperative aspects are provided in Figure 1. One young patient underwent successful heart transplantation 8 months after DAH implantation due to pre-existing myocardial failure, which did not improve despite DAH implantation and normal homograft function (Figure 2A-H).

In 4 out of 5 suspected endocarditis cases (Patients 1,8,10 and 11) no specific bacteria were identified despite a thorough microbiological work-up. In one case (Patient 1) the DAH was left in situ as it did not display any pathological findings and the ascending aorta prosthesis was replaced using a xenogenic pericardial tube (Figure 1A). Intraoperative microbiological samples including PCR showed no bacterial growth. In the second case (Patient 10) a similarly unaffected DPH was removed together with the stenosed subvalvular Gore-Tex material, while a new longer DPH was selected for the redo operation to avoid the use of any artificial material for the right ventricular outflow tract reconstruction. Simultaneously, a David-procedure was performed in this patient following a former Ross operation. Intraoperative microbiological samples again showed no bacterial growth and it is possible that the stenotic alteration of the Gore-Tex graft may have been caused by aortic root dilatation (Figure 3A-H).

Patient 11 presented with an acute development of relevant aortic regurgitation five month after a regular follow-up examination at our institution. Intraoperative findings and a histological examination were conclusive for bacterial endocarditis (Figure 1C), although no specific bacteria were identified. Two months prior to clinical presentation the patient underwent a non-medical auto-hemotherapy elsewhere, which may have been a potential origin of the infection leading to cusp destruction. A mechanical valve was implanted within the homograft, which showed only mild alteration, and was followed by standard antibiotic treatment. At 12 months of follow up, the patient remains free of recurrent endocarditis.

The overall endocarditis rate for decellularized homografts, including the unclear cases described above, was calculated as 0.68% per patient-year (5+1 in 881.1 patient-years).

*Spectrum of spontaneous recellularization*

There was good agreement between the two readers as reflected by an inter-agreement weighted Kappa of 0.783 (95% CI 0.707 to 0.859) and an intra-observer agreement weighted Kappa of 0.670 (95% CI 0.502 to 0.839; 3 randomly selected patients evaluated by D.J. > 9 months after the initial analysis). Supplemental Tables 2 and 3 show the evaluation of matrix (and cusp) structure, amount and type of recellularization for each patient as graded by the two pathologists. The overall relative histological score was 60% (±16.1) compared with normal tissue. Results in non-endocarditis cases were significantly better with 76% (±4.3%,) vs. 48% (±7.3%, p=.0004 Mann-Whitney-U-Test for independent samples).

The patient with the highest histological score was an infant who had received a 10 mm DAH implantation at 6 weeks of age, as reported.[4] There was good overall recellularization of the graft with mesenchymal cells, smooth muscle cells and myofibroblasts (Figure 3A-G). Almost no immune cells were found, resulting in a score of 34 points (81%) out of a possible maximum of 42 points. Figure 3H depicts a significant amount of intracellular pro-collagen present in cells with a fibroblastic morphological appearance within the aortic sinus 4.5 years after implantation.

The lowest histological score was 41%, which was determined for two patients. One young adult female patient suffered a severe Staph. aureus endocarditis, which led to a massive infiltration by immune-competent cells and the destruction of the DPH to an extent close to perforation. The other patient, an adult patient, who received a DPH for PVR following a former Ross operation, developed progressive valvular stenosis despite pliable cusps. At explantation 12 months postoperatively, the graft diameter had shrunken, whereas the wall and cusps macroscopic appearance were almost normal (Figure 1D). The wall structure was shown to be severely compromised and there was prominent infiltration by granulocytes, T- and B-lymphocytes without any clinical signs of infection and despite negative microbiological cultures/PCR. This case was graded as a potential cell-mediated rejection and further immunological work-up initiated.

Immune system-mediated reaction was also considered in the case of a 7 year-old boy (Patient 8). After an uneventful 3-month period following DAH implantation, a rapid increase of the transvalvular gradient was observed, caused by cusp stiffening without any clinical evidence

of infection, which ultimately led to explantation of the homograft after 9 months. The macroscopic appearance of the cusps, however, suggested endocarditis as the most likely explanation due to the extensive calcification, which was supported by the histological analysis showing extensive infiltration by neutrophils and scarce bacteria (Figure 1B). Microbiological cultures and PCR were both negative.

In 2 patients with hypoplastic left heart syndrome (Patients 4 and 6), decellularized homografts were used for the augmentation of their hypoplastic aortic arches. In one patient, a DAH was used to replace the ascending aorta; in the other a DPH was used for arch reconstruction. Biopsies were taken during a scheduled Glenn operation at 9 and 7 months, respectively. Interestingly, despite normal intraoperative findings in both homografts, the pulmonary homograft's matrix was less well preserved after 7 months in the systemic position (70 vs. 79%). The inner third of the allograft wall was sparsely populated in both patients indicating a) preferred cell invasion from the adventitial side and b) that a period of several months may be needed for recellularization.

**Comment**

The longevity of any tissue-engineered heart valve, be it artificial, xenogenic or allogeneic in origin, depends on the recipient's immunogenic response, which can result in a chronic inflammatory process and subsequent structural valve disease. A low immune response, on the other hand, is a prerequisite for the anticipated invasion of non-inflammatory autologous cell populations, which may hold the potential to promote graft regeneration.

Immune systems vary considerably between species. Transferring approaches from successful animal models to humans can have dramatically negative results.[13] As things stand, there is currently no valid animal model, which would allow adequate prediction of the performance of tissue-engineered grafts in humans. However, undertaking planned heart valve biopsies in humans is not an option for obvious ethical reasons. Consequently, conclusions can only be derived in the rare cases of scheduled procedures or unplanned re-operations.

*Extent and speed of spontaneous recellularization*

This current analysis of the largest cohort published to date adds important information as it provides insights into the extent and pace of recellularization processes in decellularized matrices in a real-life setting. Grafts obtained during the first 12 months after implantation were not evenly repopulated with less recellularization in the inner parts; no difference was found between DAH and DPH as might have been expected due to the thinner wall diameters of DPH. This finding has implications for size selection, as autologous regeneration of the allogenic matrix may not be sufficient in the first 9-12 months, thereby necessitating oversizing in growing patients.

Specimens obtained after 4.5 years showed repopulation at levels of approximately 80% of a normal cell count, including documentation of intracellular pro-collagen production. While this is not definite confirmation of matrix remodeling and extracellular component production, the clinical course of Patient 2 is very promising. A 10 mm DAH implantation in early infancy allowed for myocardial recovery, adequate somatic growth and the implantation of a 17 mm DAH at the age of 4.7 years following the resection of an evolving LVOT stenosis. We also have published the case of an 8-year old girl, where there was excellent valve function 8 years after DAH implantation, including an increase of diameter and effective orifice area.[14] In our opinion, these two examples aptly demonstrate the potential of DHV.

*Immune system mediated graft failure*

One adult patient, following DPH implantation in a post Ross-procedure situation, experienced early and steadily progressive graft failure due to diameter shrinkage in the whole graft, despite the cusps indicating normal performance in echocardiography. Histological analysis showed severe damage of the matrix structure and a high number of infiltrating immune competent cells without any clinical or microbiological evidence for bacterial endocarditis. Although we did not find the classical signs of a T-cell mediated graft rejection we grade this case as a potentially immune system-mediated graft failure. We observed similar but milder clinical courses in some pediatric DPH patients, who in part required balloon valvuloplasty.[7] Da Costa also reported shrinkage of a decellularized pulmonary homograft in a pediatric patient 8 years after implantation in the absence of classical rejection signs or calcification.[15]

This indicates that despite elimination of about 99% of donor DNA during DHV processing some patients may still elicit an immune response. It is possible that remnants from the decellularization process itself may also be present and pathogenic, albeit unlikely due to the multiple washing steps after detergent decellularization. Further research should be directed towards detecting the mechanisms of such immune reactions. As fresh DHV samples become available during processing and due to recently established prolonged storage options, matching against patient serum prior to implantation presents a possible option to avoid such graft failure in the future.

*Endocarditis*

The observed risk for endocarditis in DHV is in the reported range of 0.5-1% per patient-year for different types of heart valve prostheses, which is also supported by the low endocarditis incidence reported by Da Costa for pediatric pulmonary valve replacement.[15] The risk for endocarditis using DPV for PVR is significantly lower than the reported incidence for bovine jugular vein conduits.[16] A interesting finding of our study was that 50% of the endocarditis cases were associated with prosthetic material. As there is a well-established increased risk of endocarditis when using artificial material, we recommend avoiding the use of prosthetic material in combination with DHV wherever possible as well as lifelong endocarditis prophylaxis for our patients.

*Limitations*

The results observed are likely to represent the poorer ‘performers’ within the recellularization spectrum as >80 % of the specimens were acquired in pathological situations, such as flow turbulence or infection. In addition, important aspects such as direct proof of matrix regeneration, e.g. by newly synthesized collagen integration and analysis of the extracellular substance, are lacking in our descriptive analysis, but would be extremely advantageous. Limitations are also given by the “eye-based” semi-quantitative scoring algorithm and in the specimens derived from biopsies as for obvious reasons these were not performed at leaflet levels and as such small samples do have limited information about the rest of the implanted homograft.

Finally, decellularization protocols and matrix structures can vary significantly between products and caution should therefore be applied in interpreting the results and transferring them to other decellularization techniques.[13,17]

*In conclusion*, significant in-vivo recellularization with non-inflammatory cells was observed in this study, which represents the largest human histological analysis of decellularized allografts conducted to date. Spontaneous recellularization appears to require multiple months, which correspondingly has an impact on size selection for fast growing young patients. One case of immune system mediated early graft failure was observed without the classical signs of a T-cell mediated rejection. Further research should be directed towards detecting the mechanisms of such immune reactions.

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Table 1 – patient characteristics and gross follow-up data for decellularized pulmonary and aortic valves.

| | Pulmonary valve | Aortic valve |
|---|---|---|
| Period | 01/2005-04/2017 | 02/2008-04/2017 |
| Diameter | 24.0±3.6 mm | 22.2±3.4 mm |
| Patients | 236 | 128 |
| Mean age | 18.9±12.9 yrs. | 18.7±14.2 yrs. |
| Age range | 0.1-72.9 yrs. | 0.2-65.4 yrs. |
| Follow-up | 100% (2149 exams) | 100% (665 exams) |
| Patient years total | 640 | 248.1 |
| Mean follow-up years | 2.7±3.0 yrs. | 2.12±1.9 yrs. |
| Maximum gradient (mmHg) | 20.3±15.0 | 16.0± 5.2 |
| Regurgitation (Grade 0-3) | 0.70±0.6 | 0.50±0.5 |
| Freedom from explantation | 98.7% (n=3) | 96.1 % (n=5) |

Table 2 – Clinical information and histological score for each specimen

| | Type of specimen | Reason for re-intervention | Age at implant in years | Diagnosis | Implant duration in months | **Overall relative histological result** 42 points were classified as 100 %, mean value of Pathologist 1+2 |
|---|---|---|---|---|---|---|
| Patient 1 | Biopsy on distal aortic homograft anastomosis | Suspected infection of ascending aorta vascular prosthesis | 53 | Aortic stenosis | 14 | 45% |
| Patient 2 | Explanted aortic homograft | Explantation due to subvalvular stenosis and regurgitation | 0.2 | Aortic stenosis | 55 | 81% |
| Patient 3 | Biopsy at supravalvular anastomosis | Supravalvular stenosis | 5 | Transposition of the great arteries (TGA) | 39 | 74% |
| Patient 4 | Aorta ascendens biopsy | Staged re-operation (Glenn) | 0.1 | Hypoplastic left heart syndrome (HLHS) | 9 | 79% |
| Patient 5 | Explanted pulmonary homograft | Severe Staphyloccus aureus endokarditis | 13 | Tetralogy of Fallot (TOF) | 60 | 41% |
| Patient 6 | Aortic arch biopsy (PA conduit) | Staged re-operation (Glenn) | 0 | Hypoplastic left heart syndrome (HLHS) | 7 | 70% |
| Patient 7 | AVR | Heart transplantation due to preexisting myocardial damage | 2.4 | Shone complex | 8 | 75% |
| Patient 8 | AVR | Severe cusp immobility | 7 | Aortic stenosis | 9 | 52% |
| Patient 9 | PVR | Developing valvular stenosis | 43 | S/P Ross | 12 | 41% |
| Patient 10 | PVR | Subvalvular stenosis, concomittant David-procedure | 17 | S/P Ross | 38 | 60% |
| Patient 11 | AVR | Endocarditis following non-medical autohemotherapy | 47 | Aortic stenosis | 44 | 44% |

DPH- decellularized pulmonary homograft, DAH - decellularized aortic homograft

**Figure Legends**

Figure 1: Freedom from explantation Kaplan-Meier curves for decellularised pulmonary and aortic homografts delineating the fraction of non-explanted homografts at the respective follow-up year after implantation.

Figure 2: (A) shows the intraoperative status of the decellularized aortic homograft (DAH) of Patient 1, who was operated due to suspected endocarditis of the aorta ascending vascular prosthesis. The DAH was unaffected and left in-situ. (B) shows severe cusp calcification due to endocarditis, Patient 8. (C) Perforation of the non-coronary cusp of Patient 11 (insert), while the other cusps where translucent and pliable. (D) Shrunken decellularized pulmonary homograft of Patient 9 with thickened graft wall and preservation of cusp function.

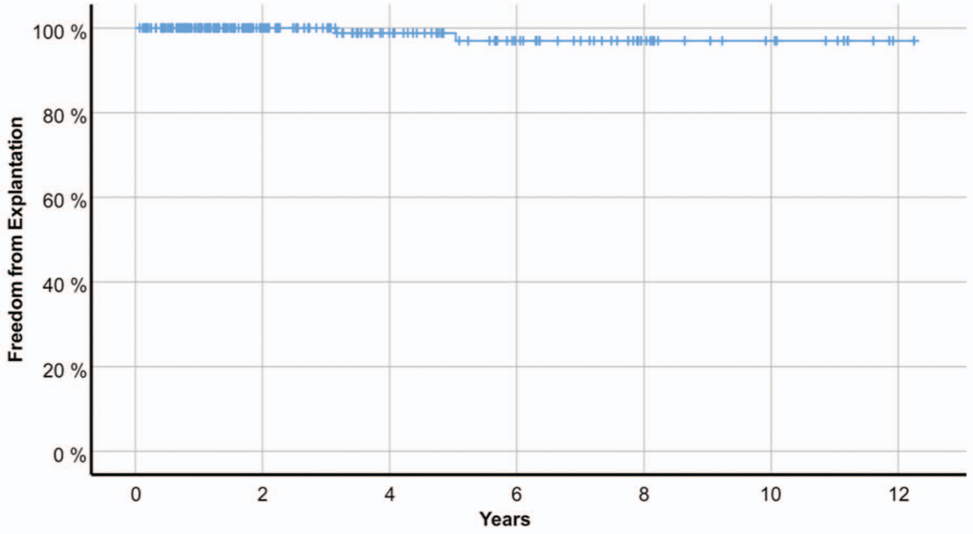
Figure 3: (A) Intraoperative findings 8 months after DAH implantation in a 2.4 year girl, HTX due to pre-existing myocardial failure despite normal graft function. (B)Elastica van Gieson staining (EvG) of the whole graft in longitudinal direction, the white arrow indicates the area of Figure 2D. (C)Smooth muscle α-actin staining (SMA) of the wall, as indicated in 2B. (D) T-lymphocyte staining (CD3) of the wall as indicated in 2B.

Figure 4: (A) Cardiac magnetic resonance imaging aspect of Patient 10 showing RVOT stenosis in the area of a Gore-Tex vascular graft subvalvular to a non-stenotic decellularized pulmonary homograft (DPH). The pulmonary autograft in aortic position shows significant dilatation. (B) HE staining of the immediate neighbouring DPH to the Gore-Tex prosthesis. Inserts specify the region of the magnified views. (C)SMA staining for smooth muscle cells and myofibroblasts. (D) Granulocyte (CD15) staining.

Figure 5: (A) Intraoperative aspect 4.5 years after DAH implantation in a 0.2 year-old boy, explantation due to subvalvular stenosis and valvular regurgitation. (B) Haemotoxylin and Eosin staing(HE) of the non-coronary sinus, the white arrow indicates the area of Figures 3C-G. (C) Vimentin staining for mesenchymal cells of the non-coronary sinus as indicated in 3B. (D) B-lymphocyte staining (CD20) staining of the non-coronary sinus as indicated in 3B.

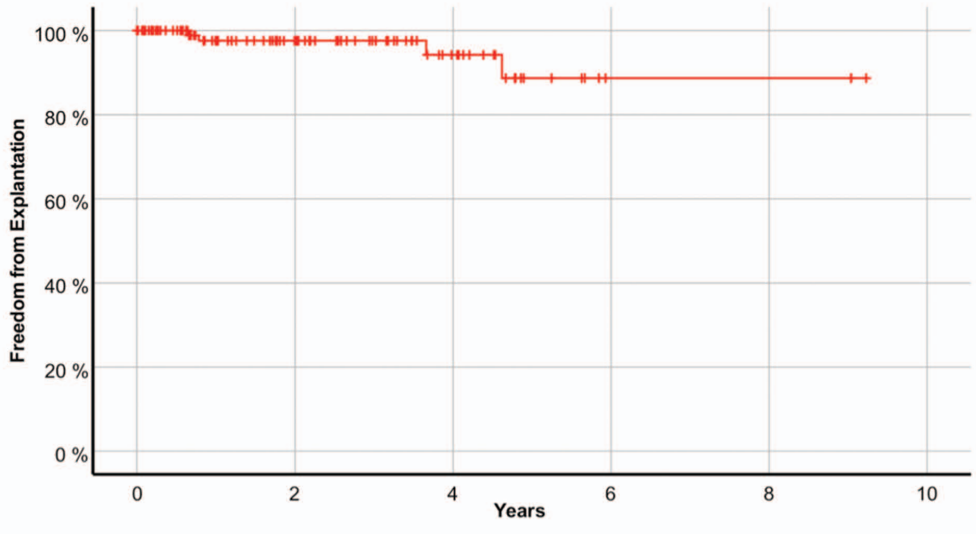
Figure 6: Overview of the non-coronary sinus repopulated with cells 4.5 years after DAH implantation in a 0.2 year old boy. Stained in HE (A) and Movat´s pentachrome staining (B), Intracellular pro-collagen type I depicted in red and DAPI nucleus-staining depicted in blue in different levels (i-iv) as indicated in B.

## Decellularised pulmonary homografts

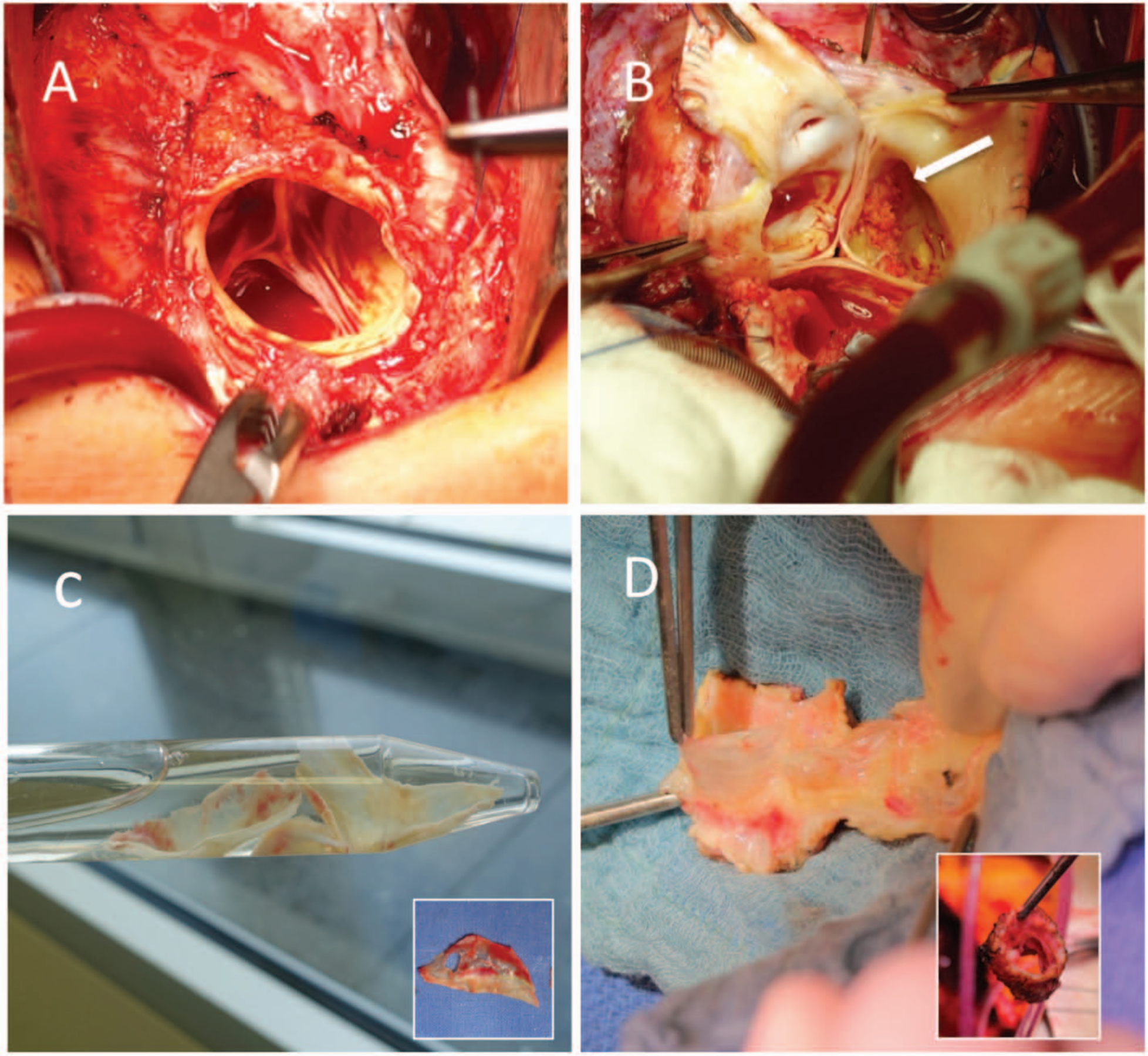


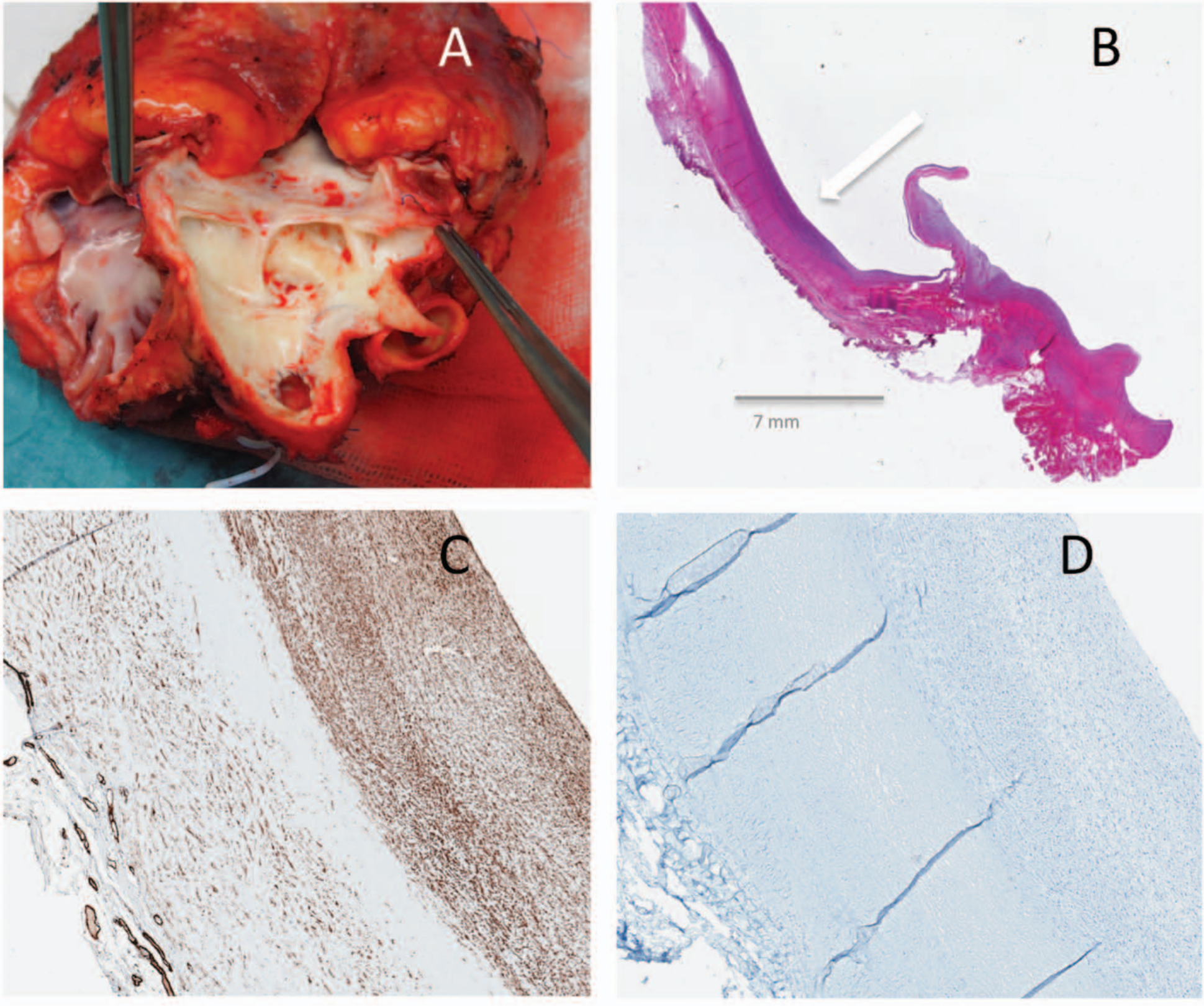

| At risk | | | | | | |
|---|---|---|---|---|---|---|
| 236 | 111 | 68 | 43 | 24 | 13 | 2 |

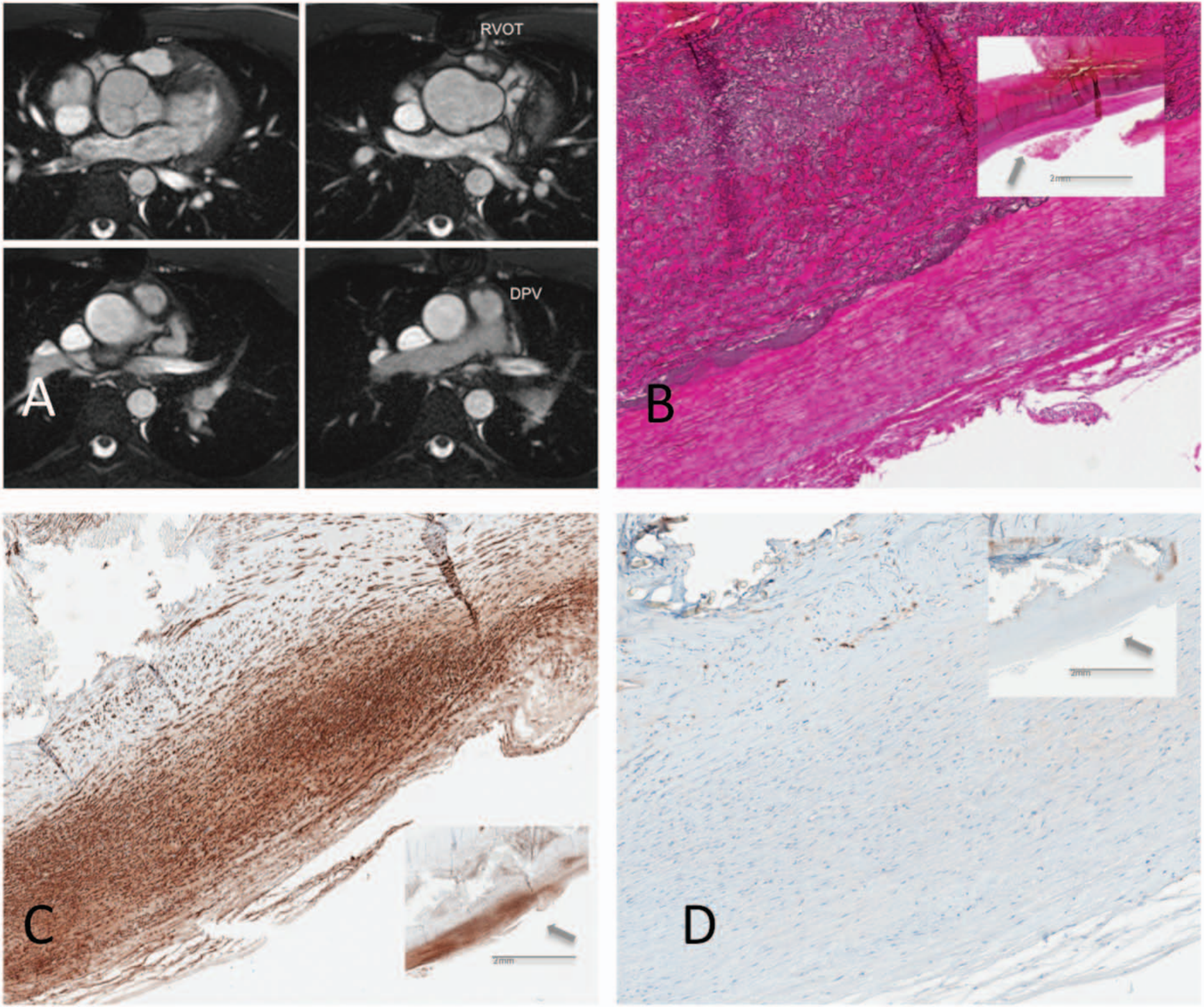
## Decellularised aortic homografts

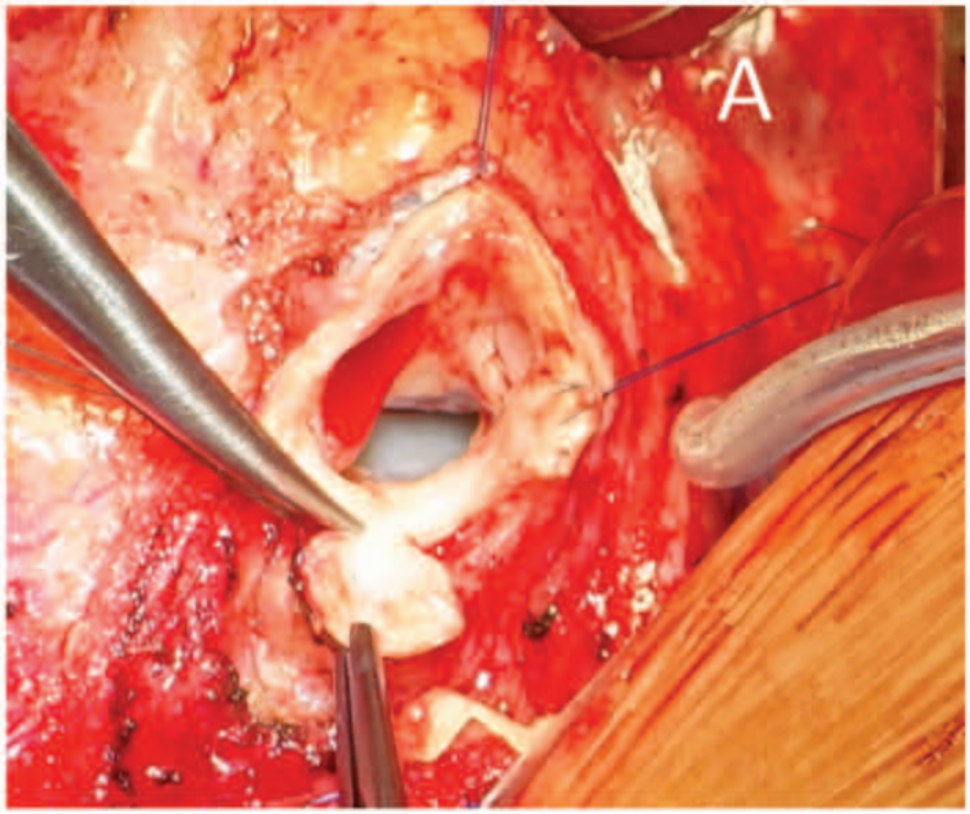


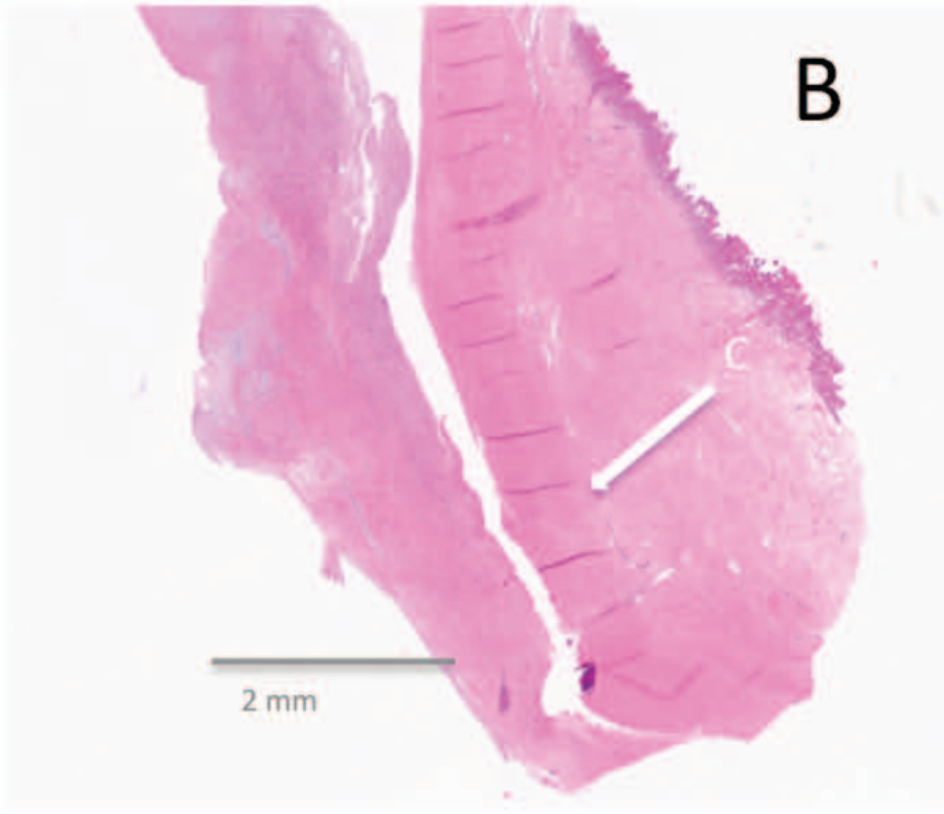

| At risk | | | | | |
|---|---|---|---|---|---|
| 128 | 57 | 24 | 3 | 3 | 2 |

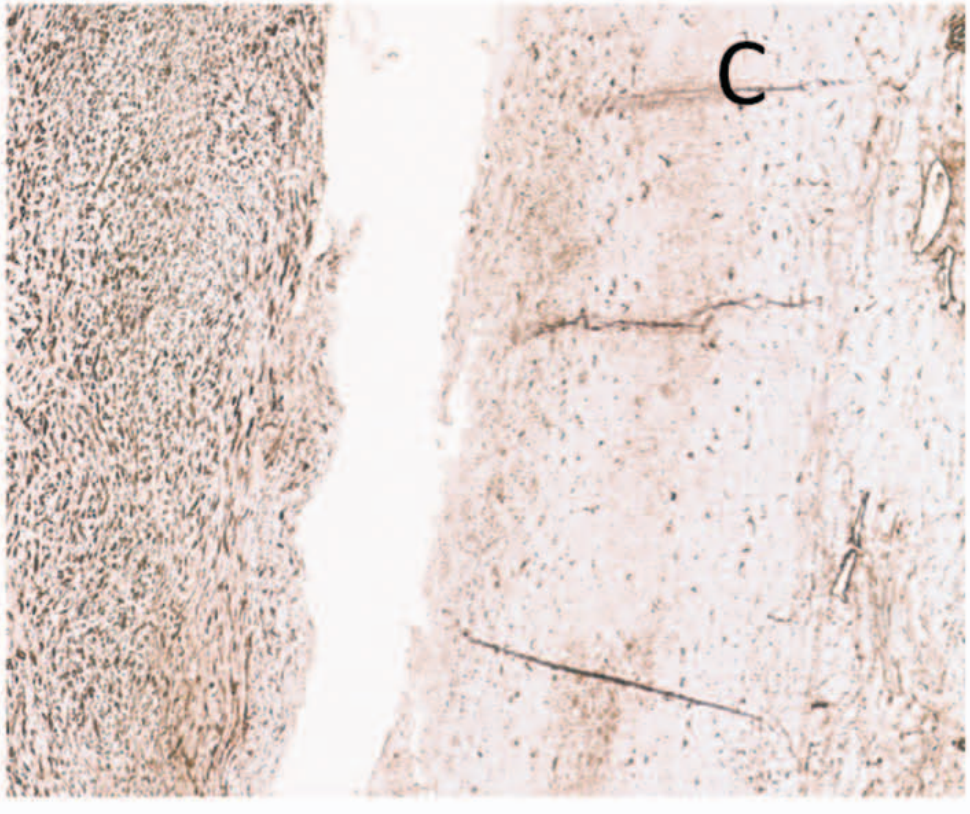

A
B
C
D


A
B
7 mm
C
D


RVOT
DPV
A
B
2mm
C
2mm
D
2mm


A


B
2 mm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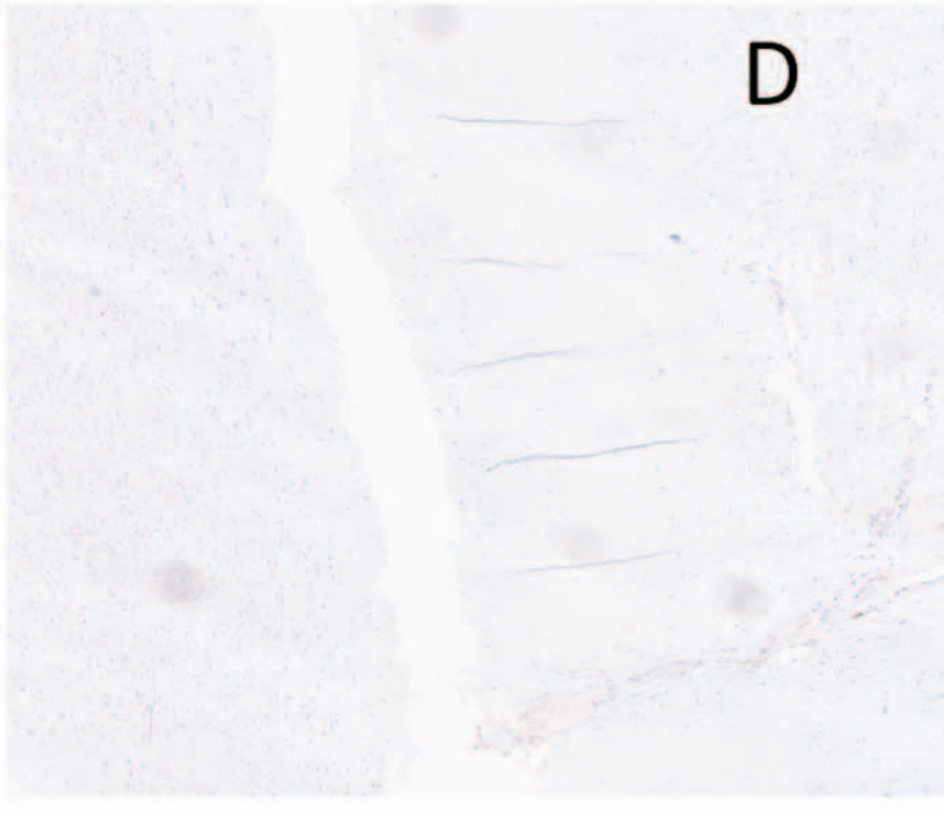
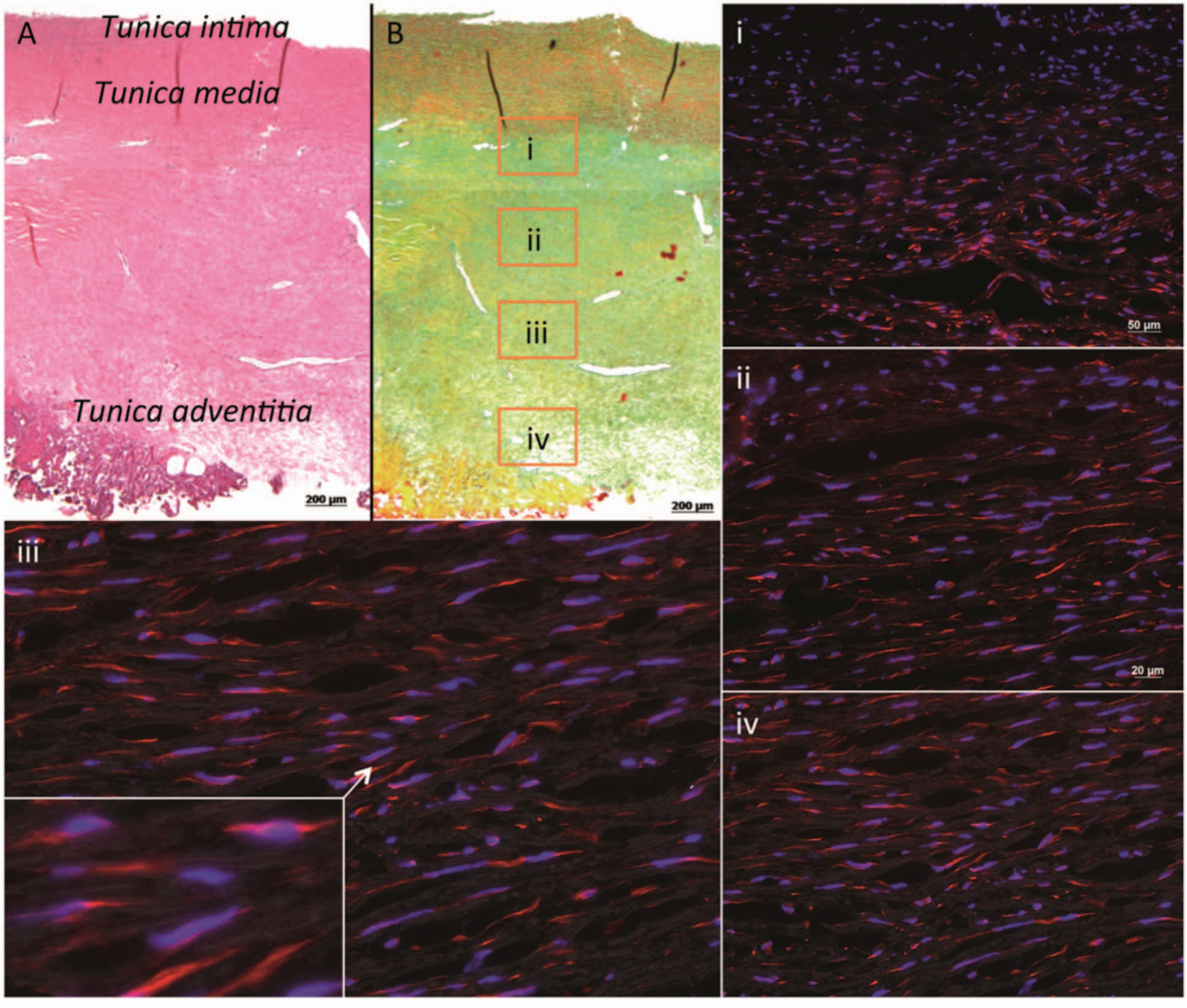

D


A
Tunica intima
Tunica media
Tunica adventitia
200 µm
B
i
ii
iii
iv
200 µm
i
50 µm
ii
20 µm
iii
iv