Early Insight Into In Vivo Recellularization of Cell-Free Allogenic Heart Valves

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

**Methods.** Two experienced cardiac pathologists independently evaluated human specimens obtained during reoperation between December 2010 and April 2017 DHVs in seven categories after automated staining (scores: 0 to 6) in comparison with published data. An optimal result of 42 points was classified as 100%.

**Results.** We found that 364 DHVs, 236 decellularized pulmonary homografts (DPHs), and 128 decellularized aortic homografts (DAHs) were implanted, and freedom from explantation was 96.1% (DAH) and 98.7% (DPH). Reoperations were because of (suspected) endocarditis in 5 of 11 patients, stenosis at the subvalvular or valvular or supravalvular level in 3 of 11 patients, planned staged reoperation in 2 of 11 patients, and 1 heart transplantation. Good reader agreement was reflected by an interagreement weighted $k$ of 0.783 (95% confidence interval: 0.707 to 0.859). The relative histologic score in nonendocarditis specimens was 76% ± 4.3% (maximum 81%). Intracellular procollagen type 1 production was found in recipient mesenchymal cells within the transplanted grafts. In endocarditis specimens the histologic score was significantly lower with 48% ± 7.3% (minimum 41%, $p = 0.0004$) because of leucocyte infiltration and matrix degradation. One 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 recellularization.

**Conclusions.** Substantial in vivo recellularization with noninflammatory 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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Little, however, is known about the important process of DHV recellularization by the recipient’s cell populations. Even though a generally low level of antigenicity has been demonstrated for DHV [6, 7], infiltration by neutrophils, lymphocytes, and macrophages may occur and can only be reliably assessed by histologic examination. Furthermore, true long-term durability of DHVs 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 allogenic DHVs, so far only sporadic histologic reports on decellularized grafts have been put forward in humans [8–11]. Specifics of tissue processing, methods, and results of histologic analyses albeit were heterogeneous, ranging from infiltration by inflammatory cells and severe calcification to good preservation of the matrix and widespread noninflammatory repopulation.

This study aims to establish more histologic evidence on the extent of spontaneous in vivo recellularization of detergent-based decellularized, noncryopreserved, and nonseeded 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 decellularization service after market approval for decellularized pulmonary homografts (DPHs) and decellularized aortic homografts (DAHs) (PEL.G.11766.01.1 and PEL.G.11634.01.1). Completeness of decellularization was assessed by histologic examination and DNA content for each homograft before release.

The postoperative clinical course of all patients who received either a DPH for pulmonary valve replacement (PVR) or a DAH for aortic valve replacement has been prospectively followed since January 2005. Approval was given by all local ethics committees before start of the studies, and informed consent was given by all participants or parents.

All explanted homografts from December 2010 to April 2017 were secured for histopathologic analysis. Specimens obtained during planned staged palliative operations were also harvested, when possible without performing any additional incision to the existing graft.

**Histologic Assessment**

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

Formalin-fixed and paraffin-embedded tissue was cut to 1-μm slices at a routine microtome. For standard and immunohistochemistry the cut slices were stained at a routine automated tissue-stainer (Ventana Ultra; Roche, Basel, Switzerland) according to the manufacturer’s specification. Antibody details are provided in Supplemental Table 1. Standard histologic analysis was performed with hematoxylin and eosin and Elastica van Gieson 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 immunohistologic staining to all specimens. With the use of the same scoring model we assessed endothelial cells, fibroblasts, myofibroblasts, and smooth muscle cells as characterized by Vimentin. Cell counts for smooth muscle cells and myofibroblasts, as characterized by positive smooth muscle cell z-actin was graded accordingly. Infiltration by granulocytes was characterized by CD15 staining, T lymphocytes by CD3, and B lymphocytes by CD20 staining.

A total score was calculated for each patient with the use of the results of two anatomic stainings (because not all specimens provided material for separate wall and cusp analysis) and five immunologic stainings. An optimal result in these seven 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 with SPSS 23 (IBM Corporation, Somer, NY). Summaries of the numeric data are given as means ± SD; a probability value of 0.05 or less was considered statistically significant. Inter-rater and intra-rater agreement (κ) was calculated with 95% confidence interval (CI) as reported by Fleiss [13]. κ values of 0.61 to 0.80 were considered good and 0.81 to 1.0 as very good.

No correlations between implant duration and recellularization rate were calculated because of the substantial heterogeneity of the small study cohort for age at implantation, reasons for reoperation, and DHV position.

**Results**

A total of 364 decellularized, nonseeded, and noncryopreserved homografts (236 pulmonary and 128 aortic) were implanted between January 2005 and April 2017. Follow-up was 100%, comprising 888.1 patient-years and more than 2,700 examinations. Details on patient demographic characteristics, homograft size, and overall hemodynamic performance are provided in Table 1.
Within this 12-year period eight decellularized homografts were explanted, leading to freedom from explantation of 96.1% for DAH and 98.7% for DPH (Fig. 1). One DAH was not available for standardized histologic assessment; this case that involved the rupture of the native ascending aorta because of an Aspergillus infection has been reported previously [1].

Eleven specimens (7 explants, 4 biopsies) were available for histologic assessment, 6 specimens were derived from DAHs, 5 from DPHs. Details of specimens, patient characteristics, and clinical situation are provided in Table 2.

### Reasons for Reoperation

Reasons for reoperation included scheduled reoperation in 2 of 11 patients; stenosis either at subvalvular, valvular, or supravalvular level in 3 patients, and endocarditis or suspected endocarditis in 5 patients. Intraoperative aspects are provided in Figure 2. One young patient underwent successful heart transplantation 8 months after DAH implantation because of preexisting myocardial failure, which did not improve despite DAH implantation and normal homograft function (Figs 3A–3D). In 4 of 5 suspected endocarditis specimens (patients 1, 8, 10, and 11) no specific bacteria were identified, despite a thorough microbiological workup. In one situation in patient 1 the DAH was left in situ because it did not display any pathologic findings, and the ascending aorta prosthesis was replaced with a xenogenic pericardial tube (Fig 2A). Intraoperative microbiological samples, including polymerase chain reaction (PCR), showed no bacterial growth. In the second patient (patient 10) a similarly unaffected DPH was removed, together with the stenosed subvalvular Gore-Tex material (W. L. Gore & Associates, Flagstaff, AZ), whereas 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 after 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 (W. L. Gore & Associates) may have been caused by aortic root dilatation (Figs 4A–4D).

Patient 11 presented with relevant aortic regurgitation 5 months after a regular follow-up examination at our institution. Intraoperative findings and a histologic examination were conclusive for bacterial endocarditis (Fig 2C), although no specific bacteria were identified. Two months before clinical presentation the patient underwent a nonmedical auto-hemotherapy elsewhere, which may have been a potential origin of the infection that led 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 in the paragraphs above, was calculated as 0.68% per patient-year (5 + 1 in 881.1 patient-years).

### Spectrum of Spontaneous Recellularization

Good agreement was found between the two readers as reflected by an inter-agreement weighted $k$ of 0.783 (95% CI: 0.707 to 0.859) and an intra-reader agreement weighted $k$ of 0.670 (95% CI: 0.502 to 0.839; 3 randomly selected patients evaluated by Dr Jonigk >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 histologic score was 60% ± 16.1% compared with normal tissue. Results in nonendocarditis cases were significantly better with 76% ± 4.3% versus 48% ± 7.3% ($p = 0.0004$ Mann-Whitney $U$ test for independent samples).

The patient with the highest histologic 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 (Figs 5A–5D). Almost no immune cells were found, resulting in a score of 34 points (81%) of a possible maximum of 42 points. Figure 6 depicts a substantial amount of intracellular procollagen present in cells with a fibroblastic morphologic appearance within the aortic sinus 4.5 years after implantation.

The lowest histologic score was 41%, which was determined for 2 patients. One young adult female patient had a severe Staphylococcus 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 after a former Ross operation, experienced 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 (Fig 2D).

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**Table 1. Patient Characteristics and Gross Follow-Up Data for Decellularized Pulmonary and Aortic Valves**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pulmonary Valve</th>
<th>Aortic Valve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>January 2005–</td>
<td>February 2008–</td>
</tr>
<tr>
<td></td>
<td>April 2017</td>
<td>April 2017</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>24.0 ± 3.6</td>
<td>22.2 ± 3.4</td>
</tr>
<tr>
<td>Patients, n</td>
<td>236</td>
<td>128</td>
</tr>
<tr>
<td>Age, years</td>
<td>10, and 11</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or n (%) unless otherwise specified.
The wall structure was shown to be severely compromised, and there was prominent infiltration by granulocytes, T lymphocytes, and B lymphocytes without any clinical signs of infection and despite negative microbiological cultures or PCR. This case was graded as a potential cell-mediated rejection, and further immunologic workup was 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 after 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 because of the extensive calcification, which was supported by the histologic analysis that showed extensive infiltration by neutrophils and scarce bacteria (Fig 2B). 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 1 patient, a DAH was used to replace the ascending aorta; in the other patient a DPH was used for arch reconstruction. Biopsies were taken during a scheduled Glenn operation at 9 and 7 months, respectively. Of interest, despite normal intraoperative findings in both homografts, the pulmonary homograft’s matrix was less well preserved after 7 months in the systemic position (70% versus 79%). The inner third of the allograft wall was sparsely populated in both patients, indicating (1)

Table 2. Clinical Information and Histologic Score for Each Specimen

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of Specimen</th>
<th>Reason for Reintervention</th>
<th>Age at Implant, Years</th>
<th>Diagnosis</th>
<th>Implant Duration, Months</th>
<th>Overall Relative Histologic Result, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biopsy on distal aortic homograft anastomosis</td>
<td>Suspected infection of ascending aorta vascular prosthesis</td>
<td>53</td>
<td>Aortic stenosis</td>
<td>14</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Explanted aortic homograft</td>
<td>Explantation due to subvalvular stenosis and regurgitation</td>
<td>0.2</td>
<td>Aortic stenosis</td>
<td>55</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>Biopsy at subvalvular anastomosis</td>
<td>Supravalvar stenosis</td>
<td>5</td>
<td>Transposition of the great arteries</td>
<td>39</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>Aorta ascendens biopsy</td>
<td>Staged reoperation (Glenn)</td>
<td>0.1</td>
<td>HLHS</td>
<td>9</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>Explanted pulmonary homograft</td>
<td>Severe Staphylococcus aureus endocarditis</td>
<td>13</td>
<td>Tetralogy of Fallot</td>
<td>60</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>Aortic arch biopsy (PA conduit)</td>
<td>Staged reoperation (Glenn)</td>
<td>0.1</td>
<td>HLHS</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>AVR</td>
<td>Heart transplantation due to preexisting myocardial damage</td>
<td>2.4</td>
<td>Shone complex</td>
<td>8</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>AVR</td>
<td>Severe cusp immobility</td>
<td>7</td>
<td>Aortic stenosis</td>
<td>9</td>
<td>52</td>
</tr>
<tr>
<td>9</td>
<td>PVR</td>
<td>Developing valvular stenosis</td>
<td>43</td>
<td>S/P Ross</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>PVR</td>
<td>Subvalvular stenosis, concomitant David procedure</td>
<td>17</td>
<td>S/P Ross</td>
<td>38</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>AVR</td>
<td>Endocarditis after nonmedical autohemotherapy</td>
<td>47</td>
<td>Aortic stenosis</td>
<td>44</td>
<td>44</td>
</tr>
</tbody>
</table>

Forty-two points were classified as 100%, mean value of pathologist 1 + 2.

AVR = aortic valve replacement; DAH = decellularized aortic homograft; DPH = decellularized pulmonary homograft; HLHS = hypoplastic left heart syndrome; PA = pulmonary artery; PVR = pulmonary valve replacement; S/P = status post.
preferred cell invasion from the adventitial side and (2) 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 antigenic response, which can result in a long-term inflammatory process and subsequent structural valve disease. A low immune response, however, is a prerequisite for the anticipated invasion of noninflammatory 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 [14]. As things stand, there is currently no valid animal model that 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 reoperations.

Extent and Speed of Spontaneous Recellularization
This present analysis of the largest cohort published to date adds important information because 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 because of the thinner wall diameters of DPH. This finding has implications for size selection, because autologous regeneration of the allogenic matrix may not be sufficient in the first 9 to 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 procollagen production. Although this is not definite confirmation of matrix remodeling and extracellular component production, the clinical course of patient 2 is 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 after the resection of an evolving left ventricular outflow tract stenosis. We also have published the case of an
Fig 3. (A) Intraoperative findings 8 months after decellularized aortic homograft (DAH) implantation in a 2.4-year-old girl, Heart transplantation due to preexisting myocardial failure despite normal graft function. (B) Elastica van Gieson staining of the whole graft in longitudinal direction, the white arrow indicates the area of Figure 3D. (C) Smooth muscle α-actin staining of the wall, as indicated in Figure 3B. (D) T-lymphocyte staining (CD3) of the wall as indicated in Figure 3B.

Fig 4. (A) Cardiac magnetic resonance imaging aspect of patient 10 showing right ventricular outflow tract (RVOT) stenosis in the area of a Gore-Tex (W. L. Gore & Associates, Flagstaff, AZ) vascular graft subvalvular to a nonstenotic decellularized pulmonary valve (DPV). The pulmonary autograft in aortic position shows substantial dilatation. (B) Hematoxylin and eosin staining of the immediate neighboring DPV to the Gore-Tex prosthesis. Inserts specify the region of the magnified views. (C) Smooth muscle α-actin staining for smooth muscle cells and myofibroblasts. (D) Granulocyte (CD15) staining. (B–D: scale bar = 2 mm.)
8-year-old girl in whom there was excellent valve function 8 years after DAH implantation, including an increase of diameter and effective orifice area [15]. In our opinion, these two examples aptly demonstrate the potential of DHVs.

**Immune System-Mediated Graft Failure**

One adult patient, after DPH implantation in a situation after Ross procedure, experienced early and steadily progressive graft failure because of diameter shrinkage in the whole graft, despite the cusps indicating normal performance in echocardiography. Histologic 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 classic signs of a T-cell–mediated graft rejection, we did grade this case as a potentially immune system-mediated graft failure. We observed similar but milder clinical courses in some pediatric patients with DPH, who in part required balloon valvuloplasty [7]. Da Costa and colleagues [16] also reported shrinkage of a DPH in a pediatric patient 8 years after implantation in the absence of classic rejection signs or calcification.

This indicates that, despite elimination of approximately 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 because of the multiple washing steps after detergent decellularization. Further research should be directed toward detecting the mechanisms of such immune reactions. As fresh DHV samples become available during processing and because of recently established prolonged storage options, matching against patient serum before implantation presents a possible option to avoid such graft failure in the future.

**Endocarditis**

The observed risk of endocarditis in DHV is in the reported range of 0.5% to 1% per patient-year for different types of heart valve prostheses, which is also supported by the low endocarditis incidence reported by Da Costa and colleagues [16] for pediatric PVR. The risk of endocarditis with the use of DPH for PVR is significantly lower than the reported incidence for bovine jugular vein conduits [17]. An interesting finding of our study was that 50% of the endocarditis cases were associated with prosthetic material. Because 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...
because more than 80% of the specimens were acquired in pathologic situations, such as flow turbulence or infection. In addition, important aspects such as direct proof of matrix regeneration (eg, by newly synthesized collagen integration and analysis of the extracellular substance) are lacking in our descriptive analysis, but they would be extremely advantageous. Limitations are also given by the eye-based semiquantitative scoring algorithm and in the specimens derived from biopsies, because, 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 greatly between products, and caution should therefore be applied in interpreting the results and transferring them to other decellularization techniques [14, 18].

Conclusion
Substantial in vivo recellularization with noninflammatory cells was observed in this study, which represents the largest human histologic 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 classic signs of a T-cell–mediated rejection. Further research should be directed toward detecting the mechanisms of such immune reactions.
The authors wish to thank Nina McGuinness, ELS, for editorial assistance.

Axel Haverich holds shares in corlife oHG, the company that processed the decellularized allografts used in this study. Hannover Medical School is not a shareholder of corlife oHG and does not participate in its success.

This study was supported by two grants from the European Union’s Seventh Framework Program under Grant Agreement No. 278453 (ESPOIR) and the Horizon 2020 Research and Innovation Program under Grant Agreement No. 643597 (ARISE).

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