

Diplomarbeit

**Alpha-Gal specific humoral immune
response after implantation of bioprostheses
in cardiac surgery**

zur Erlangung des akademischen Grades

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Wien, März 2010

Andreas Mangold

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13.1 Letter to the Editor, accepted in *Journal of Heart and Lung Transplantation* 92

Every year, more than 100.000 US patients need to have their dysfunctional or diseased valves replaced with a prosthetic valve. Where there is a need, there is a technological solution. The heart valve industry in the US is vibrant and healthy, enjoying a growth in the market of 5% per year, selling roughly 300.000 valves worldwide. Worldwide sales were \$910 million in 2002 and are most likely past the \$1 billion mark in 2005. Faced with such tremendous market opportunities, many companies, clinicians, and scientists alike have taken serious interests in developing a new type of heart valve that can potentially revolutionize the industry and the practice of medicine.

— <http://wwwp.medtronic.com/newsroom>, November 2009 (Medtronic Inc; Minneapolis, USA)

1 ABSTRACT

Background: It has been shown that the α -Gal (Gal α 1.3-Gal β 1-4GlcNAc-R) epitope is a relevant xenoantigen present on bioprostheses utilized in cardiac surgery and elicits an α -Gal specific IgM immune response [2]. The aim of this diploma thesis is to investigate whether that immune response proceeds after valve implantation.

Materials and Methods: Plasma samples were collected from patients who underwent bioprosthesis implantation (n=19) or mechanical valve replacement (n=8), prior to, at ten days and at three months after cardiac surgery. ELISA was performed to quantify α -Gal specific IgG and IgG subclasses. Three bioprosthetic tissue samples were obtained from patients who passed or had to undergo reoperation within one week (n=1) or at 12–15 months (n=2) after the initial operation. Confocal laser scanning microscopy (CLSM) was utilized to detect the presence of α -Gal epitopes (IB4) and cell nuclei (DAPI).

Results: α -Gal specific IgG was significantly increased three months after implantation of bioprostheses compared to preoperative values (p<0.001) and was significantly higher than α -Gal specific IgG levels of the control group (p<0.05). IgG3 was the major subclass directed against α -Gal (p<0.05, pre- vs. postoperative values). CLSM analysis demonstrated that bioprostheses explanted one week after implantation contained IB4/DAPI positive cells within the collagen matrix. In contrast, in patients who underwent reoperation after 12 months, porcine tissue showed a complete lack of IB4/DAPI.

Conclusion: These results indicate that the implantation of bioprostheses elicits a specific humoral immune response against α -Gal bearing cells compared to controls within three months after cardiac surgery. The complete absence of IB4/DAPI positive structures 12 months after implantation indicates a specific degradation of α -Gal bearing cells through previous exposure to the human blood circuit.

2 ZUSAMMENFASSUNG

Hintergrund: 2005 konnten *Konakci et al.* zeigen, dass α -Gal (Gal α 1.3-Gal β 1-4GlcNAc-R) auf bioprothetischen Herzklappen vorhanden ist und eine α -Gal spezifische IgM Immunantwort auslöst [2]. Das Ziel dieser Diplomarbeit ist es, zu untersuchen, ob diese Immunantwort nach der Klappenimplantation fortschreitet.

Material and Methoden: Serumproben wurden von Patienten, die biologische (n=19) oder mechanische (n=8) Herzklappen erhielten, vor, zehn Tage nach und drei Monate nach Klappenimplantation entnommen. Um die α -Gal spezifischen IgG und IgG-Subklassen Antikörper zu bestimmen, wurde die ELISA-Technik angewandt. Gewebeproben solcher biologischen Klappen wurden von Patienten entnommen, die eine Woche (n=1) oder 12 Monate (n=2) nach der Klappenimplantation verstorben sind oder nochmals operiert werden mussten. α -Gal Epitope (IB4) und Zellkerne (DAPI) wurden angefärbt und mittels konfokalem Laser-Scan Mikroskop dargestellt.

Ergebnis: α -Gal spezifisches IgG war drei Monate nach der Operation im Vergleich zu präoperativen Werten signifikant erhöht ($p < 0,001$), und war außerdem signifikant höher als die entsprechenden IgG-Titer der Kontrollgruppe ($p < 0,05$). IgG3 war bei dieser Immunreaktion die dominante IgG-Subklasse. ($p < 0,05$, prä- vs. postoperative Werte). Mit CLSM konnte gezeigt werden, dass IB4/DAPI-positive Zellen in der Kollagenmatrix von nach einer Woche explantierten Klappen vorhanden sind. Demgegenüber zeigten die nach 12 Monaten explantierten Klappen keinerlei IB4- oder DAPI-positive Strukturen.

Diskussion: Diese Resultate zeigen, dass die Implantation von Bioprothesen eine spezifische Immunantwort gegen α -Gal auch drei Monate nach der Operation auslöst. Durch die völlige Abwesenheit von IB4/DAPI-positiven Strukturen 12 Monate nach Implantation scheint es wahrscheinlich, dass diese Strukturen durch die kontinuierliche Exposition im Blutkreislauf spezifisch abgebaut wurden.

3 INTRODUCTION

3.1 Prosthetic heart valves

3.1.1 History of valve surgery

The early years

The commencements of cardiac surgery are to be found at the turn of the last century. The problem of heart valve repair or replacement had not been approached until general operation and anaesthetic techniques (cardiopulmonary bypass, cardioplegia, etc.) were established. The first successful experiment replacing a heart valve of a dog with an artificial prosthesis was performed by *Rehmi Denton* in New York in 1949 [3]. *Gordon Murray* made promising attempts in replacing stenotic mitral valves with venous grafts in humans in the late forties [4]. *Charles Hufnagel* carried out good approaches with ball valves in the descending aorta position [5]. As early as then, two different mind-sets were established, some pursuing biological valve grafts, others developing artificial prosthesis. In May, 1960, *Dwight Harken* inserted valves consisting of a lucite ball in a stainless-steel cage in the aortic position [6]. At the same time *Albert Starr* and *Lowell Edwards* turned up on the scene. They also produced ball-cage valves and put much effort in the manufacturing process [7]. These valves should turn out to be the major choice for valve replacement (VR) in the next years (Fig.3.1.). Nevertheless, VR stayed restricted to otherwise hopeless cases.

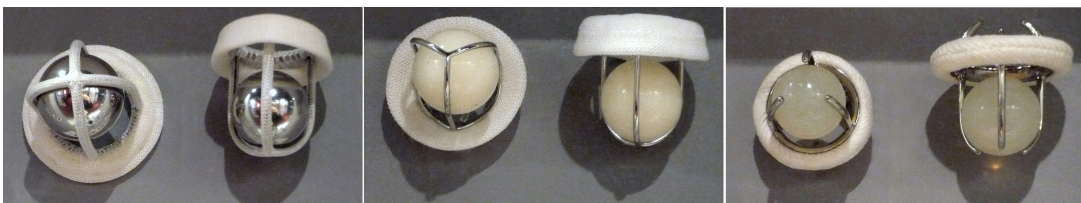


Figure 3.1: Starr-Edwards Ball Cage Valves ©Copyright 2008, Dr. Mirko Junge

In parallel, the development of bioprostheses proceeded. Different grafts were tried out: autografts, homografts and xenografts. In 1952, *Robert Litwak* performed experiments in which he replaced aortic valve tissue with homologous valve tissue for the first time [8].

The replacements functioned well, but fibrosed all and lost function after some time. In 1956, *Murray* reported a case in which he inserted a homograft aortic valve in the descending aorta [9]. In the next years, people did not pay too much attention to homografts until it was realized that *Murray's* first patient was still alive six and a half years later [10].

Donald Ross had also been studying valve homotransplantation, and implanted his first freeze-dried homograft in 1962 during an operation with valvotomy complications. The patient received anticoagulants and cortisone and recovered very well [11]. By 1964, he had operated on 11 more patients. After treating some patients, he recognized that there is no rejection in any patient, so he stopped the cortisone therapy [12]. At the same time, *Brian Barratt-Boyes* in New Zealand operated with a similar technique on more than 40 patients, most of them with a very good outcome [13].

Due to the scarceness of homologous valve material, the question of xenologic transplantation emerged soon. The first try was given by *Duran* and *Gunning* in 1965. They transplanted porcine aortic valves into 17 dogs. They died a few months later, but the valves were still in good condition [14]. In the same year, they inserted pig aortic valves, which had been stored in mercurochrome antiseptic into human patients. None of them received anticoagulants or immunosuppressives, nevertheless they had uncomplicated recoveries [15].

More difficulties had to be overcome in replacing the mitral valve. The ball-cage valves took much space in the left ventricle and caused flow disturbances, and the insertion of biological mitral valves was troubling, as the chordae tendinae had to be set on function to prevent cusp prolaps [10]. *Hubka* from Bratislava came up with the simple idea to implant the aortic valve in upside-down manner in the mitral position and performed experiments with dogs [16]. Soon thereafter, *Ionesco* and *Whooler* in Leeds used this technique only slightly different in human patients [17].

Long term results got available, the biological valves failed to function after some time due to degeneration and calcification of the tissue. In respect to that problem, *Ross* thought about autologous transplantation and invented the Ross-OP, which had a renaissance in the last years. Thereby the patients' own pulmonary valve is placed in the aortic position, and a donor valve is inserted into the pulmonary position, where a malfunction of the valve does not have such a grievous effect and due to lower pressure conditions potentially occurs later [18].

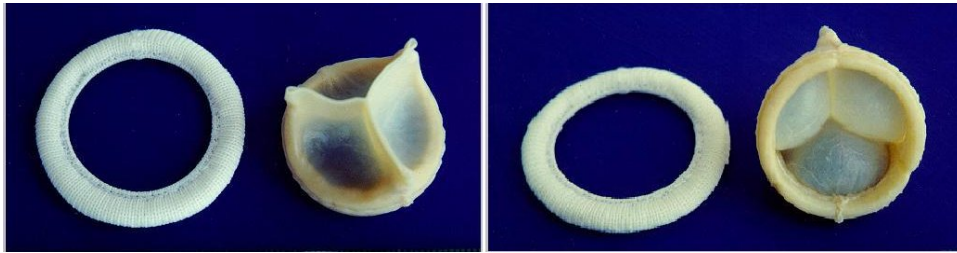


Figure 3.2: Biological Valves ©GNU Free Documentation License

The repeated impact of the steel ball on the cage struts of the ball–cage valves led to erosion and finally dysfunction. Other materials such as silicon did not meet the requirements as well [19, 20]. Thus, new strategies such as disc prostheses were pursued. The most remarkable under these was the Björk–Shiley tilting–disc prosthesis, invented in 1969 [21]. Popularity and widespread usage of the Björk–Shiley valve ended abruptly when the latest model fractured after some years in few patients [19, 22]. The next landmark of mechanical prostheses was the St. Jude pyrolytic carbon disc prostheses of 1977 (Fig.3.3.) [23]. The triumphal success of that design is still going on and has been adopted by many manufacturers.



Figure 3.3: St. Jude Pyrolytic Carbon Disc Prosthesis, with kind permission of Wuth, Peter, ©St. Jude Medical Austria

3.1.2 Further development until present

Progress of biological valve engineering was made, when new preservation and processing techniques came up. Properties of homografts were highly improved with the introduction of cryopreservation by *O'Brian and associates* in 1987 [24]. Even more important was the successful fixation of xenografts with glutaraldehyde (GA), which greatly extended the durability by *Carpentier et al.* Trials with formaldehyde did not show this effect, as they failed to function after few years [25].

The introduction of stentless xenograft valves brought both more challenge for the surgeon and more benefit for the patient. Insertion time is 10 to 30 minutes longer compared to stented valves [26], but the transvalvular pressure gradient is reduced to 5–6 mmHg. The left ventricular mass is more reduced in aortic valve replacement (AVR) than with stented grafts at 6 months in most trials. Long term beneficial effects of stentless xenograft valves are still under discussion [27, 28, 29].

Valve tissue engineering

Most effort in the research field deals with new processing steps of the valves or tissue engineering. Several methods have been tested in the last decades, with mostly restrained and sometimes disastrous outcomes. Approaches that have been tested in the past years [30]:

Decellularization of xenogenic tissue This approach aims to remove all cells from valve tissue, leaving only the collagen matrix, with or without reseeding other cells afterwards. Detergents used were anionic Sodium dodecyl sulfate, the zwitterionic CHAPS and CHAPSO, and the nonionic BigCHAP, Triton X-100, and Tween family of agents [30]. The idea is easy to comprehend. Firstly, antigenicity of xenograft valves is expected to be reduced if the cells are removed. Secondly, homograft valves are only cryopreserved, but not processed any further (no crosslinking via GA as in xenografts). These homografts become completely acellular after weeks to months in the circulation [31]. The life span of those valves is up to 20 years. So why not decellularizing xenografts and getting the same effect?

Pitifully, results differed from theory. In a trial in Vienna, decellularized valves have been implanted in neonates with congenital valvular malformations who had few alternatives. After the operation, many of these children developed serious valvular complications, several died. The pathohistological analysis showed severe inflammation, both inside and out, fibrosis, encapsulation, perforation, and deterioration of the leaflet tissues [32].

At least since that trial, researchers focussed on seeding cells into the valve matrix before implantation [33]. Up to now, no convincing results could be reached with that neither. Instead of settling and revitalizing the valve matrix, the cells rather seem to diminish the mechanics of the microstructure [34, 35]. A currently popular approach is the use of stem cells, endothelial progenitor cells for example, to seed in the scaffolds. Results are very preliminary, future will reveal whether this concept is convincing [36, 37].

Use of bioresorbable synthetic scaffolds The idea is to seed cells on a porous material. This scaffold is implanted in the organism; there, the cells shall generate the organ while the scaffold degenerates. Some progress has been made in the last years, but in spite of promising short-term results, major obstacles such as fibrosis, retraction and incompetence hamper wider usage [38, 39].

Collagen-based constructs containing entrapped cells That concept addresses the observation that cells entrapped in collagen gels contract and compact the gels, increasing the density of the collagen manifold [40, 41]. Experiments using reconstituted collagen as a substrate for tissue engineering have failed because cells entrapped in collagen gels rapidly enter apoptosis and synthesize matrix metalloproteinases [42]. Therefore, other agents such as chitosan mixed with collagen [43] or hybrid concepts using hyaluron are being tested [44].

Percutaneous and minimally invasive valve replacement

Since a few years, surgeons are trying to develop techniques to offer solutions to valve diseased patients who are unsuitable for median sternotomy and cardiopulmonary bypass. People get older, treatments are constantly improving, and comorbidities are more and

more emerging. According to the Euro Heart Survey, a third of the elderly patients are not referred for surgery by their doctors in private practise [45]. Established techniques for those patients are minimal invasive incisions, percutaneous VR, transapical VR and some other, less frequently performed concepts. Advantages of minimal invasive techniques are less postoperative pain, improved cosmetics, less blood loss, fewer pulmonary and wound complications, psychological comfort, and shorter length of stay [46, 47, 48, 49, 50, 51]. Nevertheless, anaesthesia, cardiopulmonary bypass and cardioplegic arrest are necessary for these approaches, therefore benefit is moderate. Methods tried out to overcome this problem are PORT ACCESS [52], transapical AVR [53] and ventriculuaortic valved conduit, the latter being only suitable for aortic stenosis [54]. However, biggest hopes are lying within percutaneous VR. The valve is usually sewed in a stent which is placed in the descending aorta. The antegrade implantation involves transseptal puncture, flotation of the balloon through the left atrium, ventricle and the aortic valve; this is extremely complex and has been largely abandoned in favor of the retrograde approach, entering through the femoral artery. The applications in clinical trials are listed in Table 3.1.

3.1.3 Morbidity and Mortality related to bioprosthetic and mechanical heart valves

When a valve-diseased patient requires surgical therapy, several decisions have to be made. The preferred operation technique for diseases of the mitral valve are commisurotomy, valvotomy and valve repair; replacement is only performed if no other method is suitable. For aortic valve diseases, replacement is the most common treatment [56]. Indications for valve replacement therapy are listed under 3.5. When valve replacement is indicated, the type of valve has to be chosen. The major question is whether a biological or a mechanical valve is favored. The general difference has already been mentioned: Mechanical prostheses almost always have an unlimited durability, but require life-long anticoagulation therapy. Bioprostheses do not require anticoagulation, but have a restricted durability. Bioprostheses conclude autografts, homografts and xenografts. Autografts show an excellent performance and endure for a long time period. Therefore, the Ross operation technique is very common in infant cardiac surgery [57]. Homografts perform better than xenografts, but their availability is limited. As xenograft valves are the most often implanted grafts, the term “bioprostheses” refers to these valves in the

Valve Lesion	Approach	Device	Clinical Experience	Advantages/ Disadvantages
Aortic stenosis	Balloon valvuloplasty		Extensive	Poor durability
	Valve replacement	Cribier–Edwards valve	Phase I	Antegrade approach: complex (simplified retrograde approach)
		Core Valve	Phase I	Improved design features
Mitral regurgitation	Edge-to-edge repair	E valve	Phase II	Primarily indicated for leaflet prolapse
	Annuloplasty Cardiac Dimensions	Carillon	Phase I	Coronary sinus approach: redesigned
		Edwards Monarc	Phase I	Coronary sinus approach: redesigned
		Viacor	Phase I	Coronary sinus approach
	Transventricular	Coapsys	Phase II	Indicated for annular dilatation/ functional MR
Mitral stenosis	Balloon valvuloplasty		Extensive	Effective

Table 3.1: Selected Percutaneous Approaches Specific to Valvular Pathology [55]

following discussion. A major consideration is the patients' age, as it is well known and could be often reproduced that valve deterioration of bioprostheses occurs faster in young recipients. Objective studies did not find significant difference between certain established models used [58, 59].

Hammermeister et al. (Final Report of the Veterans Affairs Randomized Trial [60]) studied 575 randomized patients (AVR or mitral valve (MV) replacement) prospectively who received either mechanical (Bjork–Shiley spherical disc mechanical prosthesis) or biological (Hancock porcine bioprosthetic valve) heart valves; the follow-up was 15 years. This is the longest prospectively observed time period. Other studies conducted prospective surveys up to ten years, with comparable results for the respective time frame [61, 62]. Overall-mortality in the Veterans Affairs Randomized Trial in AVR was higher in the biovalve group ($66\pm 3\%$ vs. $79\pm 3\%$, $p=0.02$), in MV replacement was no significant difference observed. Primary valve failure occurred predominantly in the bioprostheses cohort

(AVR: $23 \pm 5\%$ vs. 0% , $p=0.0001$, MV replacement: $44 \pm 8\%$ vs. $5 \pm 4\%$, $p=0.0002$ Fig.3.4.) and therein mainly in patients <65 . In patients >65 , primary valve failure was not significantly different between bioprostheses and mechanical valves (AVR: $9 \pm 6\%$ vs. 0% , $p=0.16$).

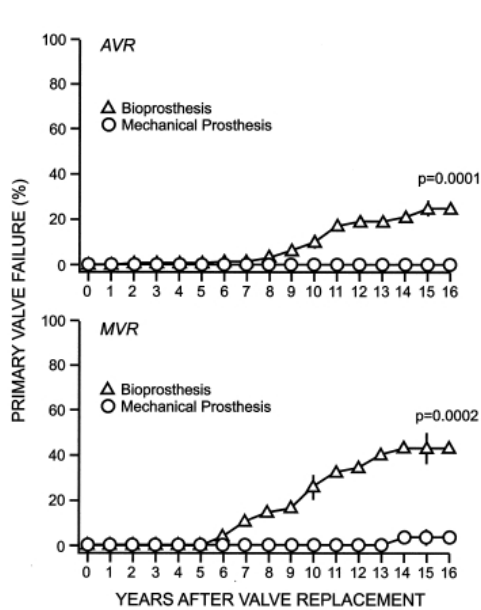


Figure 3.4: Primary valve failure (non-thrombotic valve obstruction or central valvular regurgitation), adapted from Hammermeister et al. [60]

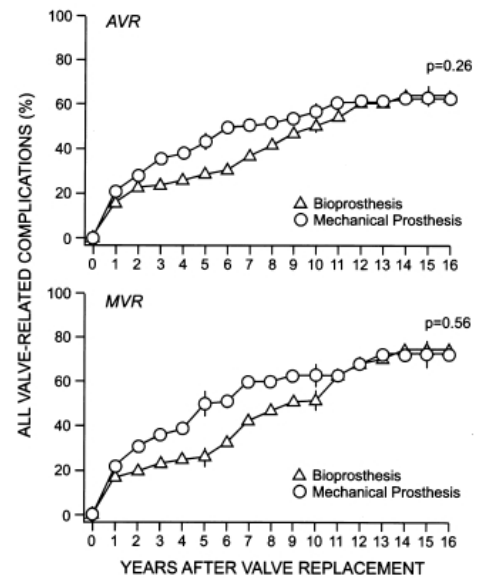


Figure 3.5: Occurrence of one or more valve-related complications (bleeding, endocarditis, systemic embolism, nonthrombotic valve obstruction, valvular regurgitation or valve thrombosis), adapted from Hammermeister et al. [60]

Reoperation was significantly higher for aortic bioprostheses recipients ($29 \pm 5\%$ vs. $10 \pm 3\%$, $p=0.004$, Fig.3.6.). Bleeding emerged more often in the mechanical valve group compared to the biovalve group for both AVR ($51 \pm 4\%$ vs. $30 \pm 4\%$, $p=0.0001$) and MVR ($53 \pm 7\%$ vs. $31 \pm 6\%$, $p=0.01$, Fig.3.7.).

No significant differences for other complications between the two groups could be observed. Especially thromboembolism, which was thought to occur more often in mechanical prosthesis recipients, did not show any disparity.

The authors are qualifying the results concerning bleeding as they state that a harsh anticoagulative regimen (prothrombin time maintained at 2.0 to 2.5 times control) has

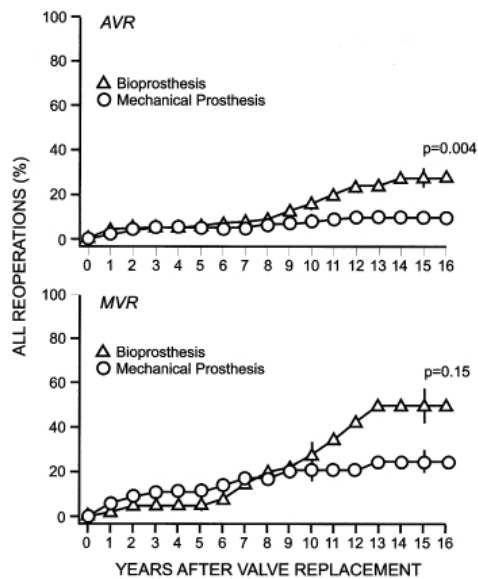


Figure 3.6: Reoperation for any reason on randomized valve, adapted from Hammermeister et al. [60]

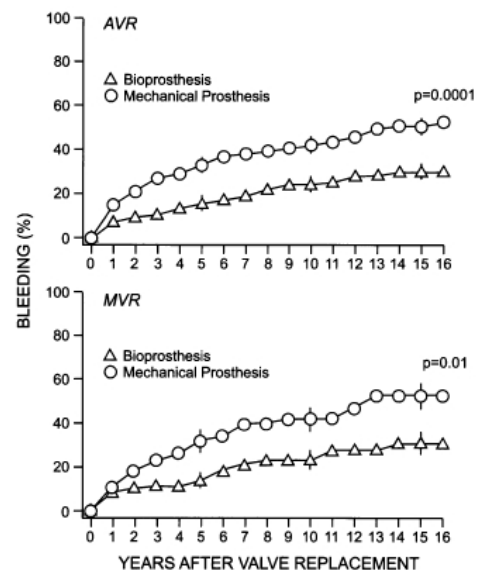


Figure 3.7: One or more clinically significant bleed(s), adapted from Hammermeister et al. [60]

been subscribed in the study. They recommend a less restrictive anticoagulation for bioprostheses recipients in clinical practise.

The strong significant difference in mortality was due to deaths after primary valve failure in the bioprostheses cohort. These deaths occurred virtually all in the 10 to 15 year time period, and predominantly in patients under 65. In patients over 65, primary valve failure was not significantly different. Similar results were found in a recent study by Stassano et al. [63].

3.1.4 Pathophysiology of bioprosthetic valve degeneration

The continuous degeneration of bioprosthetic heart valves is compounding two major factors: (a) Calcification, intrinsic within the valve tissue, extrinsic on the surface leading to deposits, thrombi and endocarditis vegetations, and (b) structural deterioration of collagen [64].

The underlying roots of this process are still not clear. In the past, experts considered fixed tissue as immunologically inert, although evidence for this assumption was missing.

This premiss has been criticized by scientists, who favored immunological explanations for valve destruction. Both sides accumulated results supporting either one side. In the nineties, this discussion even became somewhat emotional. It can be assumed that economic interests also play some role here, diverse disclosures are being published anyway.

Mineralization with calcium phosphate of valve tissue is influenced through host metabolism, implant structure, chemistry, and mechanical factors. It predominantly starts within non-viable connection tissue cells. Moreover, extracellular matrix proteins, being mainly collagen and elastin, can function as nucleation sites for calcium phosphate. Calcification occurs more intense at sites of motional stress, e.g. points of flexions in heart valves [65, 66]. *Schoen and Levy*, long-term opinion leader in this field, provide possible explanations for the calcification process: Functionally non-viable cells in fixed tissue take up calcium through their membrane-associated phosphorus, but lack their physiological extrusion mechanisms, resulting in calcium phosphate accumulation. Secondly, they propose an active dysregulated environment comparable to atherosclerosis [64]. Immunological groundings are considered not relevant, from their point of view. They performed experiments comparing wild type and athymic mice (cellular mechanisms), furthermore chamber models, in which tissue and immune cells are separated, but soluble factors in fluid can shift freely (humoral mechanisms). They did not observe any difference in calcification [67, 68].

On the opposite side, *Human* and *Zilla* reviewed evidence for an immunological rationale. GA fixation is considered as a main promoter of calcification [69, 70]. Therefore, and in order to avoid adverse effects such as cytotoxicity, low GA concentrations (0.1–0.5%) are used for biovalve fixation. In contrast, valves fixed with higher GA concentrations (2–3%) show much lesser calcification [71]. Furthermore, calcification is almost identical in xenografts and allografts, although allografts are not fixed at all [72].

Even more substantial is this practice of low GA concentration usage for the cross-linking percentage and thus for antigen masking. Cross-linking of surface proteins via GA fixation is performed to abolish immunogenicity. It is therefore obvious that lower GA concentrations result in higher antigenicity. Evidence for that is broad, *Nimni et al.* showed already in 1987, that standard GA fixation reduces antigenicity only to 59%, in contrast to 92% reduction achieved using enhanced fixation [73, 74, 75, 76]. *Vincentelli*

et al. showed that xenogenic tissue fixed in 0.65% GA calcified 35 times more in the same animal model than fixed autologous tissue [77]. *Human et al.* implanted GA fixed aortic wall tissue subcutaneously in new zealand rabbits. The tissue of one group were pre-incubated in serum from immunized rabbits before implantation. These samples displayed three fold higher calcification after three weeks [78]. This data constitute the basic link between biovalve degeneration and immune mechanisms.

Failure rates of allografts are distinctly higher than autografts, although neither one is fixed and the only difference is immunological compatibility [79, 80].

Bioprosthetic valve degeneration is highly accelerated in young individuals [81]. The reason for that is unknown. When immunological mechanisms are assumed, a simple explanation would be the much higher activity of the immune system in the young [82].

Human and *Zilla* focus on a narrow range of responses taking part in the reaction against cross-linked xenograft tissue [79]:

- mild tissue opsonization with preformed ABs
- macrophage recruitment, antigen processing, and antigen presentation to the immune system
- mild, specific IgG response to these antigens

Antigen recognition by immunoglobulins of the IgM and IgG isotype has got two major effects: opsonization for phagocytosis and enzymatic degradation, and complement-mediated cell lysis via the membrane attack complex (MAC). The IgM class is especially competent for MAC activation [83]; however, direct cell cytotoxicity and lysis, neither complement- nor cell-driven (CD8+ T cells), is likely to occur in fixed cross-linked cells of bioprosthetic valve tissue. Moreover, bound IgM enables macrophages and granulocytes to bind C1q and C5a with consecutive release of exocytotic degranulation. The acute inflammatory milieu this reaction would cause is not being seen in bioprosthetic heart valve degeneration. Therefore, it is unlikely that IgM complement activation is the pivotal factor [79]. Nevertheless, the activation of macrophages through IgM and IgG, directly via the Fc receptor and neutrophils via IgM and iC3b [84], seems to be crucial in the degeneration process.

Collagen remains unimpaired through GA fixation, and elastin is hardly accessible to cross-linking. Thus, matrix-metalloproteinases (MMPs) are the only way to degrade these proteins (MMP-1 and MMP-8 for collagen, MMP-12 for elastin). T cells preferentially express MMP-2, MMP-9 and MMP-14, but induce proteinase release in macrophages [85], which express MMP-1 and MMP-12 [86]; neutrophils express MMP-8 [87]. Actually, these cells are present in explanted bioprosthetic heart valves as can be seen in immunohistochemical images [88, 89].

The following pathophysiological process is proposed by *Human* and *Zilla*: Macrophages interact with the foreign tissue either through humoral factors (IgM, complement) or directly through a "foreign-body reaction". They take up foreign material, degrade it intracellularly and present these peptides through MHC II molecules to T-helper cells. A common activation cascade including cytokines, chemokines and recruitment of further T cells and macrophages is initiated. Activated macrophages respond with a peroxidase burst, which contributes to tissue degeneration. Activated T-helper cells on their part activate B cells and secrete IL-4, resulting in immunoglobulin class-switching from IgM to IgG. Also an T cell-independent B cell activation is possible [90]. These IgG ABs again act as opsonins and pursue valve degeneration [79].

The results of *Schoen and Levy* mentioned above [67, 68] can be put into perspective as follows: A T cell-independent humoral immune response is taking place, whereby T cell independent type 2 antigens may activate CD5+ B cells in the athymic nude mouse. *Lin et al.* demonstrated such a response in heart-transplanted athymic mice [91]. Referring to the chamber experiments, the authors may have missed the possibility of a macrophage response to the chamber itself. The chamber implanted rats displayed more calcification than the directly implanted ones, the hemichamber rats had nearly doubled calcium levels. The only explanation is that the chamber itself has got an influence on calcification [68]. And once more, it has to be emphasized that subcutaneous implantation is not sufficient to depict processes happening inside continuous blood circulation.

3.1.5 Indications for valve replacement therapy

Excerpt of the 2008 AHA Guidelines for valvular heart disease [56].

		SIZE OF TREATMENT EFFECT →			
		CLASS I <i>Benefit >>> Risk</i> Procedure/Treatment SHOULD be performed/administered	CLASS IIa <i>Benefit >> Risk</i> Additional studies with <i>focused objectives</i> needed IT IS REASONABLE to perform procedure/administer treatment	CLASS IIb <i>Benefit ≥ Risk</i> Additional studies with <i>broad objectives</i> needed; additional registry data would be helpful Procedure/Treatment MAY BE CONSIDERED	CLASS III <i>Risk ≥ Benefit</i> Procedure/Treatment should NOT be performed/administered SINCE IT IS NOT HELPFUL AND MAY BE HARMFUL
ESTIMATE OF CERTAINTY (PRECISION) OF TREATMENT EFFECT	LEVEL A Multiple populations evaluated* Data derived from multiple randomized clinical trials or meta-analyses	<ul style="list-style-type: none"> Recommendation that procedure or treatment is useful/effective Sufficient evidence from multiple randomized trials or meta-analyses 	<ul style="list-style-type: none"> Recommendation in favor of treatment or procedure being useful/effective Some conflicting evidence from multiple randomized trials or meta-analyses 	<ul style="list-style-type: none"> Recommendation's usefulness/efficacy less well established Greater conflicting evidence from multiple randomized trials or meta-analyses 	<ul style="list-style-type: none"> Recommendation that procedure or treatment is not useful/effective and may be harmful Sufficient evidence from multiple randomized trials or meta-analyses
	LEVEL B Limited populations evaluated* Data derived from a single randomized trial or nonrandomized studies	<ul style="list-style-type: none"> Recommendation that procedure or treatment is useful/effective Evidence from single randomized trial or nonrandomized studies 	<ul style="list-style-type: none"> Recommendation in favor of treatment or procedure being useful/effective Some conflicting evidence from single randomized trial or nonrandomized studies 	<ul style="list-style-type: none"> Recommendation's usefulness/efficacy less well established Greater conflicting evidence from single randomized trial or nonrandomized studies 	<ul style="list-style-type: none"> Recommendation that procedure or treatment is not useful/effective and may be harmful Evidence from single randomized trial or nonrandomized studies
	LEVEL C Very limited populations evaluated* Only consensus opinion of experts, case studies, or standard of care	<ul style="list-style-type: none"> Recommendation that procedure or treatment is useful/effective Only expert opinion, case studies, or standard of care 	<ul style="list-style-type: none"> Recommendation in favor of treatment or procedure being useful/effective Only diverging expert opinion, case studies, or standard of care 	<ul style="list-style-type: none"> Recommendation's usefulness/efficacy less well established Only diverging expert opinion, case studies, or standard of care 	<ul style="list-style-type: none"> Recommendation that procedure or treatment is not useful/effective and may be harmful Only expert opinion, case studies, or standard of care
Suggested phrases for writing recommendations ¹		should is recommended is indicated is useful/effective/beneficial	is reasonable can be useful/effective/beneficial is probably recommended or indicated	may/might be considered may/might be reasonable usefulness/effectiveness is unknown/unclear/uncertain or not well established	is not recommended is not indicated should not is not useful/effective/beneficial may be harmful

Figure 3.8: Levels of evidence, adapted from the AHA Guidelines for Valvular Heart Disease [56]

Aortic Stenosis (AS)

Class I & IIa

1. AVR is indicated for symptomatic patients with severe AS. (*Level of Evidence: B*)
2. AVR is recommended for patients with severe AS and LV systolic dysfunction (ejection fraction (EF) less than 0.50). (*Level of Evidence: C*)

-
3. AVR is indicated for patients with severe or moderate AS undergoing coronary artery bypass graft surgery (CABG), surgery on the aorta or other heart valves. (*Level of Evidence: B, C*)

AVR can furthermore be considered for asymptomatic patients if AS is severe and mal-response to exercise, and/or likelihood of rapid progression.

Adults suffering from severe, symptomatic AS only benefit from AVR. AVR should be performed as soon as possible after the onset of symptoms. Severity is defined through one of the following characteristics:

- **Jet velocity (m/s)** greater than 4.0
- **Mean gradient (mmHg)** greater than 40
- **Valve area (cm²)** less than 1.0
- **Valve area index (cm² per m²)** less than 0.6

For asymptomatic patients, the operative benefits and risks have to be compared to improvement of life quality and risks of sudden death. Currently, there is general agreement that the risk of AVR exceeds any potential benefit in patients with severe AS who are truly asymptomatic with normal LV systolic function [56].

Aortic Regurgitation (AR)

Class I & IIa

1. AVR is indicated for symptomatic patients with severe AR irrespective of LV systolic function. (*Level of Evidence: B*)
2. AVR is indicated for asymptomatic patients with chronic severe AR and LV systolic dysfunction (EF 0.50 or less) at rest and reasonable with severe LV dilatation. (*Level of Evidence: B*)

-
3. AVR is indicated for patients with chronic severe AR while undergoing CABG or surgery on the aorta or other heart valves. (*Level of Evidence: C*)

In patients with pure, chronic AR, AVR should be considered only if AR is severe. Patients with only mild AR are not candidates for AVR, and if such patients have symptoms or LV dysfunction, other causes should be considered.

Severity is defined through one of the following characteristics:

- **Angiographic grade 3 – 4+**
- **Color Doppler jet width** Central jet, width greater than 65% LVOT
- **Doppler vena contracta width (cm)** Greater than 0.6
- **Regurgitant volume (mL per beat)** Greater than or equal to 60
- **Regurgitant fraction (%)** Greater than or equal to 50
- **Regurgitant orifice area (cm²)** Greater than or equal to 0.30

Severely symptomatic patients with advanced LV dysfunction are hard to manage. After AVR, many patients will develop irreversible myocardial changes, and perioperative risk is high (approximately 10%). Nevertheless, even in patients with NYHA IV symptoms and EF less than 0.25, the high risks associated with AVR and subsequent medical management of LV dysfunction are usually a better alternative than the higher risks of long-term medical management alone [92].

Mitral Stenosis (MS)

Class I & IIa

1. MV surgery (repair if possible) is indicated in patients with symptomatic (NYHA III–IV) moderate or severe MS when 1) percutaneous mitral balloon valvotomy is unavailable, 2) percutaneous mitral balloon valvotomy is contraindicated because of left atrial thrombus despite anticoagulation or because concomitant moderate to severe MR is present. (*Level of Evidence: B*)

-
2. Symptomatic patients with moderate to severe MS who also have moderate to severe MR should receive MV replacement. (*Level of Evidence: C*)
 3. MV replacement is reasonable for patients with severe MS and severe pulmonary hypertension (pulmonary artery systolic pressure greater than 60 mm Hg) with NYHA I–II symptoms who are not considered candidates for percutaneous mitral balloon valvotomy or surgical MV repair. (*Level of Evidence:C*)

MV replacement is an accepted surgical procedure for patients with severe MS who are not candidates for surgical commissurotomy or percutaneous mitral valvotomy. If there is significant calcification, fibrosis, and subvalvular fusion of the MV apparatus, commissurotomy or percutaneous balloon valvotomy is less likely to be successful, and MV replacement will be necessary. Severity is defined through one of the following characteristics:

- **Mean gradient (mmHg)** Greater than 10
- **Pulmonary artery systolic pressure (mmHg)** Greater than 50
- **Valve area (cm²)** Less than 1.0

MV replacement can be performed with a risk of less than 5% in young, healthy persons, but increases to 10–20% in older patients.

Mitral Regurgitation (MR)

Class I & IIa

1. MV surgery is recommended for the symptomatic patient with acute severe MR. (*Level of Evidence: B*)
2. MV surgery is beneficial for patients with chronic severe MR and NYHA II–IV symptoms in the absence of severe LV dysfunction (severe LV dysfunction is defined as EF less than 0.30) and/or end-systolic dimension greater than 55 mm. (*Level of Evidence: B*)

-
3. MV surgery is beneficial for asymptomatic patients with chronic severe MR and mild to moderate LV dysfunction, EF 0.30 to 0.60, and/or endsystolic dimension greater than or equal to 40 mm. (*Level of Evidence: B*)
 4. MV surgery is reasonable for asymptomatic patients with chronic severe MR, preserved LV function, and new onset of atrial fibrillation or pulmonary hypertension. (*Level of Evidence: C*)

Isolated MV surgery is not indicated for patients with mild or moderate MR. MV repair is recommended over MV replacement in the majority of patients with severe chronic MR who require surgery. Severity is defined through one of the following characteristics:

- **Angiographic grade 3 – 4+**
- **Color Doppler jet area** Vena contracta width greater than 0.7 cm with large central MR jet
- **Doppler vena contracta width (cm)** Greater than or equal to 0.70
- **Regurgitant volume (mL per beat)** Greater than or equal to 60
- **Regurgitant fraction (%)** Greater than or equal to 50
- **Regurgitant orifice area (cm²)** Greater than or equal to 0.40
- **Left atrial and/or ventricular size**

Severe tricuspid Regurgitation (TR)

When normal tricuspid valves develop dysfunction, the resulting hemodynamic abnormality is almost always pure regurgitation.

Class IIa

1. Tricuspid VR is reasonable for severe TR secondary to diseased/abnormal tricuspid valve leaflets not amenable to annuloplasty or repair. (*Level of Evidence: C*)

3.1.6 Criteria for valve selection

Aortic valve surgery

Class I & IIa

1. A mechanical prosthesis is recommended for AVR in patients with a mechanical valve in the mitral or tricuspid position. (*Level of Evidence: C*)
2. A bioprosthesis is recommended for AVR in patients of any age who will not take warfarin or who have major medical contraindications to warfarin therapy. (*Level of Evidence: C*)
3. Patient preference is a reasonable consideration in the selection of aortic valve operation and valve prosthesis. A mechanical prosthesis is reasonable for AVR in patients under 65 years of age who do not have a contraindication to anticoagulation. A bioprosthesis is reasonable for AVR in patients under 65 years of age who elect to receive this valve for lifestyle considerations after detailed discussions of the risks of anticoagulation versus the likelihood that a second AVR may be necessary in the future. (*Level of Evidence: C*)
4. A bioprosthesis is reasonable for AVR in patients aged 65 years or older without risk factors for thromboembolism. (*Level of Evidence: C*)
5. Aortic valve re-replacement with a homograft is reasonable for patients with active prosthetic valve endocarditis. (*Level of Evidence: C*)

Mitral valve surgery

Class I & IIa

1. A bioprosthesis is indicated for MV replacement in a patient who will not take warfarin, is incapable of taking warfarin, or has a clear contraindication to warfarin therapy. (*Level of Evidence: C*)

-
2. A mechanical prosthesis is reasonable for MV replacement in patients under 65 years of age with longstanding atrial fibrillation. (*Level of Evidence: C*)
 3. A bioprosthesis is reasonable for MV replacement in patients 65 years of age or older. (*Level of Evidence: C*)
 4. A bioprosthesis is reasonable for MV replacement in patients under 65 years of age in sinus rhythm who elect to receive this valve for lifestyle considerations after detailed discussions of the risks of anticoagulation versus the likelihood that a second MV replacement may be necessary in the future. (*Level of Evidence: C*)

3.2 Alpha-Gal

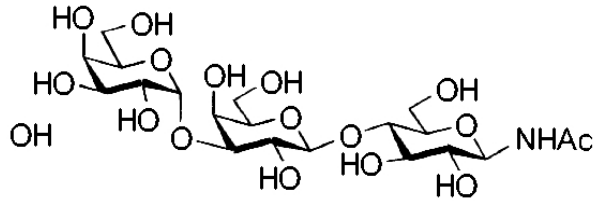


Figure 3.9: Structure of Gal α 1.3-Gal β 1-4GlcNAc-R, adapted from [93]

The α -Gal epitope (Gal α 1.3-Gal β 1-4GlcNAc-R) is synthesized through β -galactosyl α 1-3-galactosyltransferase (α 1,3GT) in an enzymatic reaction displayed in Fig.3.9. α 1,3GT is a membrane bound protein, the α -Gal epitope is generally part of the glycocalyx of mammalian cells and bacteria [1]. If there is a special function of the α -Gal epitope, is for the most part unknown; α -Gal knock-out mice suffer from cataracts, indicating it could be relevant in cell-matrix interaction [94]. Furthermore, these mice have impaired glucose tolerance [95].

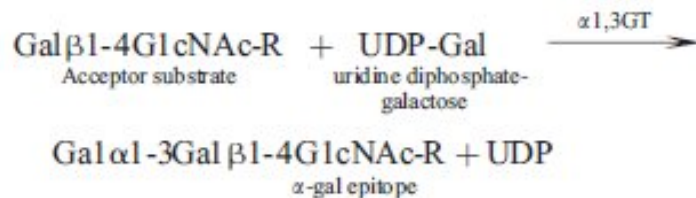


Figure 3.10: Synthesis of α -Gal, adapted from [1]

3.2.1 History

In 1968, *Eto et al.* described a glycolipid (ceramid pentahexoside) with the sequence Gal α 1.3-Gal β 1-4GlcNAc-R (Fig.3.8.) for the first time [96]. This structure has further

been characterized by several groups. In 1979, its presence on bovine cells has been reported [97, 98]; moreover on kidney tissue from pig, cow, rabbit, sheep and rat [99], and on thymus tissue of pig, sheep and rabbit [100]. Consecutive reports demonstrated various other sites of α -Gal distribution. In literature, this epitope got known as “ α -Gal” and gained more and more importance in the following years.

In 1984, *Galili et al.* discovered a specific antibody (AB) which is ubiquitarily existent in human beings and accounts for about 1% of all circulating ABs. This ABs revealed to be specific for α -Gal (Fig.3.10.) [101]. Subsequently, *Galili and co-workers* dealt extensively with α -Gal and its AB and produced a wide range of information. Apart from its most important implication, xenotransplantation, which will be described later, they explored the origin, biological functions and clinical relevancies.

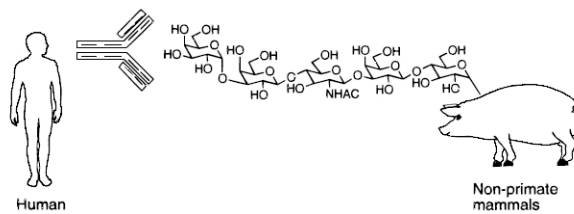


Figure 3.11: Interaction Antibodies – Alpha-Gal [102]

3.2.2 Evolution of Alpha-Gal and its Antibody [1]

The α -Gal epitope occurs in mammals, but in no other vertebrates. This indicates it is an evolutionary “young” gene. It was inactivated in Old world primates (monkeys and apes in Europe, Africa and Asia), but stayed conserved in New World monkeys. South America separated from Africa about 35 million years ago; lemurs in Madagascar, which dispersed from Africa even about 60 million years ago, also display the Gal epitope. This indicates that the inactivation of α 1,3GT was restricted to certain geographical boundaries. The main hypothesis explains this inactivation resulted from an infectious agent, most likely a virus, which was endemic in the Old World and expressed the α -Gal epitope. Thus, primates lacking this epitope and in contrast expressing the anti-Gal AB, would have had an evolutionary advantage in fighting this infection and therefore had an evolutionary advantage.

Another explanation could be that a pathogen used α -Gal as a cellular docking receptor, just like enterotoxin A of clostridium difficile. It is believed that this happened shortly before the divergence of apes and monkeys 28 million years ago [103].

3.2.3 Current knowledge

Anti- α -Gal-ABs are abundantly present in the human circulation. *Galili et al.* described IgG directed against α -Gal for the first time. Since then, specific forms of every other iso-type has been reported as well. The concentrations of these ABs are enormous: Anti-Gal IgG and IgM ABs are the most abundant specific ABs in circulation as they count for 1–2% of all circulating ABs [101, 104]. α -Gal-specific IgA has also been identified [105]. The interaction of anti-Gal IgA and bacteria in the intestine has not been investigated yet, but surely would provide useful information for tolerance and immunological homeostasis.

The reason for these high values is thought to be found in the commensal flora of the gut. *Galili et al.* showed in 1988 that several bacterial strains belonging to the commensal human gut flora express the α -Gal epitope [106]. The high concentrations of anti-Gal ABs are therefore explained through constant antigenic stimulation at the mucosal site of the intestine. Recently, this concept has been doubted by *Dahl et al.* [107]. They performed a simple and demonstrative experiment with the α -Gal knockout (KO) mouse, showing that these mice do not develop anti-Gal ABs spontaneously, although the commensal flora is α -Gal-positive and does not differ to control mice, which express the AB. Anti-Gal ABs can be induced by immunization in these mice though. They speculate that anti-Gal ABs could be rather induced by α -Gal-bearing viruses than by the gut flora.

The α -Gal ABs are closely related to AB0 ABs. α -Gal is similar to the blood group A and B antigen. Interestingly, anti-Gal ABs from blood group A and 0 carriers do react with the blood group B antigen; 85% of the anti-B ABs of these individuals are anti-Gal ABs in fact. In contrast, blood group B or AB carriers display the same anti-Gal concentrations, which obviously do not cross-react with their blood group B antigen, and

neither with the blood group A antigen. Thus it is apparent that the anti-Gal AB clones are not identical in all humans but most likely react with different sites of the α -Gal epitope [108].

Furthermore, *Galili et al.* showed that anti-Gal IgG ABs bound to senescent erythrocytes and thus enabled phagocytosis of these aged cells. They speculated that red blood cells express a similar form of α -Gal in their senescence which react with anti-Gal ABs [109].

3.2.4 Clinical Implications

The natural antigenicity of α -Gal is being used in several entities. The immune response resulting from influenza vaccination is unspecific and modest, due to the small amount of haemagglutinin and its annual changes [110]. Improvement could be achieved through targeting vaccines with highly immunogenic epitopes such as α -Gal. Vaccines would be specifically recognized by ABs and consecutively phagocytized and processed by antigen-presenting cells (APC). This has been performed in α -Gal KO mice, resulting in a 100-fold higher anti-flu AB response and largely reduced mortality [111, 112].

Similar approaches are used to establish HIV vaccinations [113] and vaccinations in tumour therapy [114]. One way to turn tumour cells immunogenic is performed using α -Gal glycolipids *in situ*. These glycolipids form into micelles when dissolved in water. When injected into solid tumours, they spontaneously insert into the tumour cell membranes. Anti-Gal ABs recognize those cells, activate complement and uptake by APCs. Through that, local obliteration of the tumour and immunisation against tumour-specific antigens, which potentially leads to removal of micrometastases, can be achieved. This could be shown in the murine model [115].

It has been suggested that anti-Gal ABs could play a role in the pathogenesis of Graves' disease, as thyroid cells express a similar form of the α -Gal epitope [116], but the results of subsequent investigations were controversial [117, 110].

Recent surveys revealed that there are individuals positive for α -Gal-specific IgE ABs. These ABs are potent to cause clinical symptoms such as anaphylaxis, angioedema and urticaria after the consumption of red meat [118].

α -Gal liposomes are effective in accelerating wound healing of skin burns. The hypothesis is simple: α -Gal epitopes are recognized by anti-Gal ABs and lead to inflammation, cytokine secretion and chemotaxis. Topical administration in α -Gal-KO mice resulted in faster and more distinct recruitment of neutrophils and macrophages in the burned lesion, and epidermis regenerated up to 100% faster than control burns [119].

An interesting diagnostic application for measuring anti-Gal-ABs has been proposed by *Galili et al.* The seminal fluid is free from anti-Gal ABs. Blood genital tract barrier damage is a possible cause of infertility in men. If such damage occurs, anti-Gal ABs trespass into seminal fluid. They can be easily detected and a broken blood barrier can be diagnosed [120].

Mosedale et al. described IgD ABs specific for α -Gal for the first time in an exploration determining anti-Gal titers in atherosclerosis [121]. α -Gal-specific IgA, IgG2 and IgM were significantly elevated in the atherosclerosis cohort. Several limitations of the survey were discussed; hypothesis explaining these higher titers remain to be investigated.

3.2.5 Xenotransplantation

Greatest impact had α -Gal and appendant ABs on the issue of xenotransplantation. The shortage of donor organs always kept up efforts to make use of animal organs, which could replace diseased organ function. Pigs are regarded as a suitable organ donor due to size, function and feasibility. Because of substantial differences in immunological relevant antigens, xenogenic grafts are rejected within minutes. This obstacle is still not overcome yet. The idea of xenotransplantation is not new. Ever since, mankind wondered about chimeric techniques. Serious trials to transplant animal organs into human beings started

in the beginning of the 20th century. In the following years, multiple attempts have been made, none being exceedingly successful. Human trials ended definitely with the fatal death of Baby Fae in 1984, after receiving a heart from a baboon [122]. Nevertheless, research proceeded, and finally *Galili et al.* identified the major source of humoral rejection: α -Gal.

Gal epitopes on animal tissue are recognized by human natural α -Gal-ABs. This activates complement, which causes cell lysis. Antibody dependent cellular cytotoxicity (ADCC) occurs, in which IgG ABs direct effector cells like macrophages and natural killer (NK) cells towards α -Gal-bearing cells. Furthermore, in xenograft blood vessels platelets are activated and aggregate within the organ, resulting in occlusion, ischemia and rejection [123, 124]. α -Gal is not the only xenoantigen, many others have been identified. In view of the high concentrations of anti-Gal ABs, these antigens are of minor relevance though [125].

Homograft transplants are protected by immunosuppressives, inhibiting immune functions on the cellular and humoral level. Sufficient suppression of xenograft rejection is not achievable with immune suppression, as necessary treatment regimen would not be reasonable [126].

Another serious issue on xenotransplantation is the transmission of zoonoses on the organ recipient. In view of the patients' impaired immune system, these zoonoses, especially porcine endogenous retrovirus (PERV), could appear and be lethal. Results on that topic report a very low probability though [127].

Beneath usual organs needed for transplantation such as heart, kidney and liver there are also attempts to use xenogenic crucial ligaments or pancreatic islet cells for treatment [128, 129].

Innumerable publications deal with xenotransplantation, and the problem is well understood. It has been tried to find strategies to solve the barrier between species, starting with the simple idea to bind Gal-ABs in serum through infusion of the disaccharide Gal α 1-3Gal or usage of affinity columns, performed in monkeys [130, 131]. This deferred hyperacute rejection for hours and days; but as soon as anti-Gal ABs were produced again, hyperacute rejection occurred.

The successful knockout of α -Gal (α 1,3GT) in mice was a landmark in xenotransplantation research. This on the one hand showed feasibility and gave insights on the physiological function, and on the other provided a powerful tool for further research. These KO mice showed no impairment except for the development of cataracts, which is related to its likely property to have some function in the glycocalyx for cell-cell interaction [94].

The aim to produce α -Gal-KO pigs was the next logical step. This goal was reached in 2002 by *Lai et al.* with the production of α 1,3GT KO pigs [132]. In these pigs, the knockout of the α 1,3GT gene eliminates the production of α -Gal. Consecutive trials with these pigs, in which kidneys and hearts were transplanted into monkeys, showed that hyperacute rejection could be restrained. Nevertheless, xenograft organs were rejected within weeks to months [133, 134, 135]. Now, although the Gal-obstacle, which counted for the most intense rejection, has been overcome, successful xenotransplantation is still far-off [136]. Of course almost every porcine peptide and every carbohydrate could act as an antigen, and against many of them ABs are present in human serum, called 'anti-non-Gal ABs' in literature.

Many other strategies for preventing xenograft rejection have been studied: immunosuppression, preformed natural AB depletion, immunomodulation, immunological tolerance and genetic manipulation [137].

One attempt was to remove gram-negative bacteria in order to deprive the source of α -Gal stimulation via antibiosis. This emerged to be much more effective than immunosuppression as anti-Gal AB production ceased for several weeks until bacteria developed resistance and recurred [138]. Although this simple idea is interesting from the scientific point of view, it will hardly be a realistic strategy in xenotransplantation.

Another was the costly production of transgenic human decay accelerating factor (hDAF) pigs, a protein that inhibits complement activation in humans [139]. This was not successful, and that approach has been largely abandoned [140].

3.3 Previous Work [2]

In 2005, *Konakci, Bohle et al.* hypothesized that a 'xenograft rejection-like' process could play a role in the degradation of GA-fixed bioprosthetic heart valves. Therefore, they investigated the presence of α -Gal-epitopes on native and fixed porcine valves using confocal laser scanning microscopy (CLSM). Despite the fixation process, α -Gal was still observable within the valve tissue (Fig.3.11. and 3.12.).

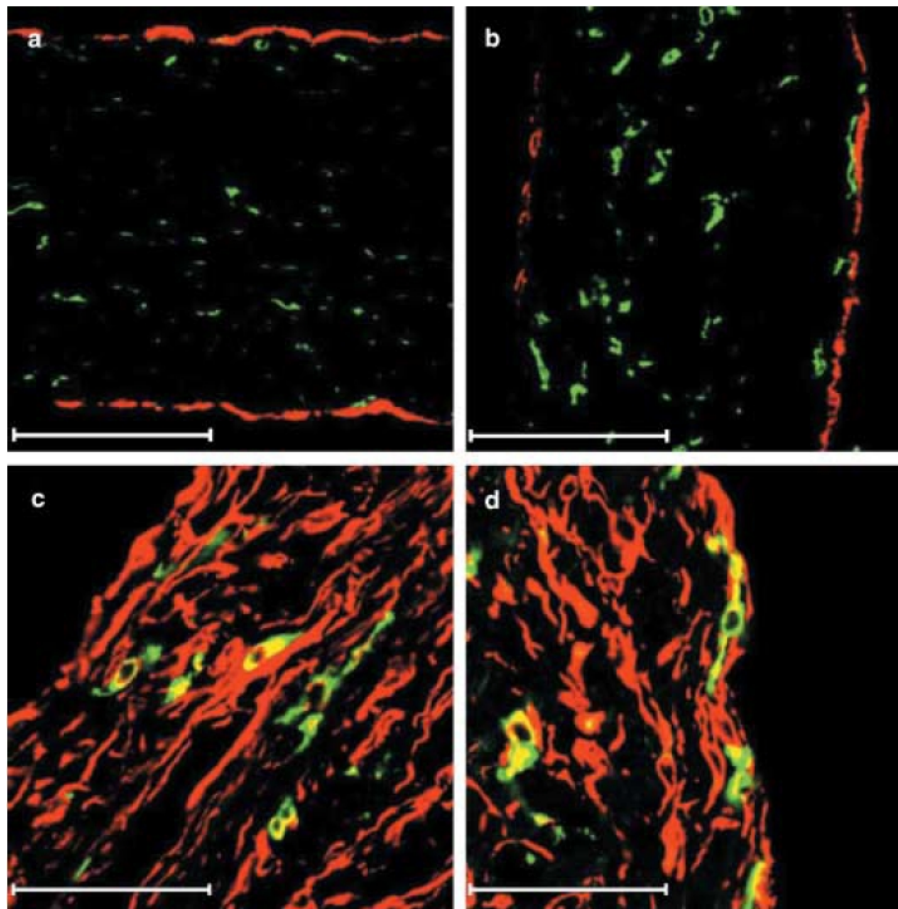


Figure 3.12: Von Willebrand factor (red) and IB4 (green) on unfixed (a) and paraformaldehyde-fixed (b) porcine valves [confocal laser scanning microscopy (CLSM) images]. The endothelial cells of the valve is stained positive for anti von Willebrand factor and shows no α -Gal expression. In the connective tissue of the valves IB4-positive cells are visible. Scale bar: 100 μ m. Vimentin (red) and IB4 (green) on unfixed (c) and paraformaldehyde-fixed (d) porcine valves (CLSM images). Numerous fibrocytes, which are stained with antivimentin, express α -Gal. Other fibrocytes exhibit no α -Gal expression. Scale bar: 50 μ m. Adapted from [2]

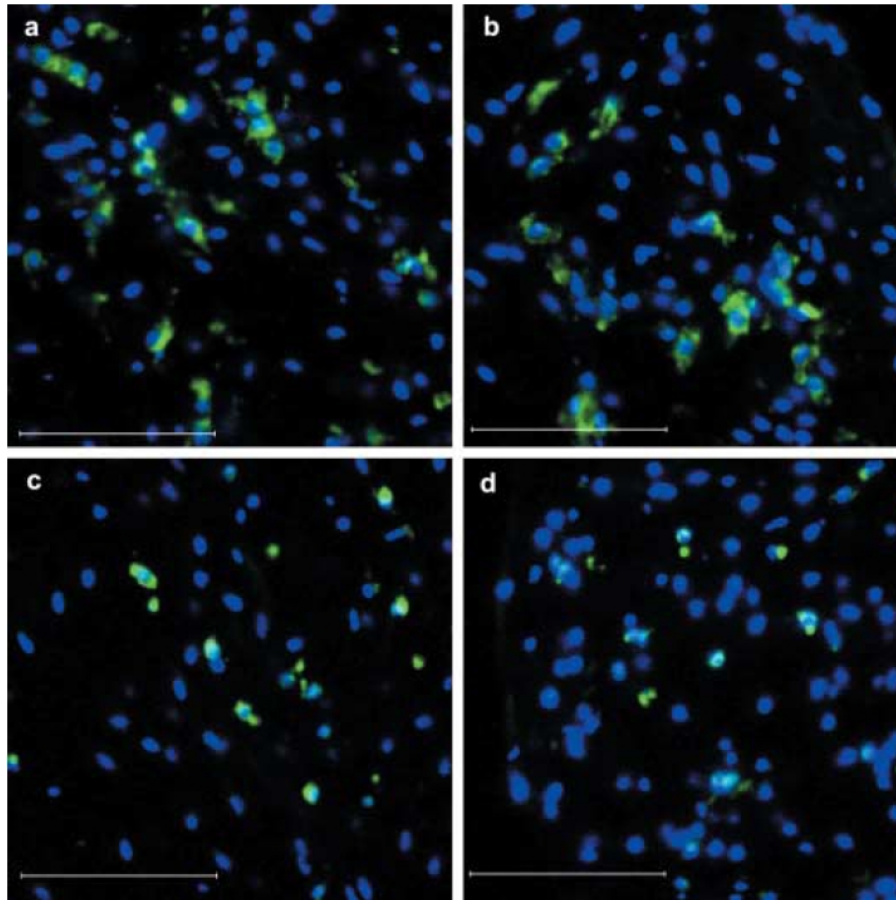


Figure 3.13: IB₄ (green) and Dapi (blue)-labelled industrially manufactured pig valves (CLSM images). Valves are from Medtronic (a: Mosaic, b: Freestyle 995, c: Freestyle 995MS) and Edwards Life Sciences (d: Edwards Prima). Numerous IB₄-positive cells are shown, all with a DAPI-positive nucleus. Scale bar: 100 μ m. Adapted from [2]

They further asked whether the implantation of bioprostheses in the human heart elicits an augmentation in α -Gal-specific IgM ABs. Blood sera were collected from biovalve recipients before and ten days after valve replacement (n=12). Recipients of mechanical valves and coronary artery bypass grafting (CABG) patients served as controls. It could be shown that specific IgM ABs increased significantly ten days after operation compared to controls (Fig.3.13.).

To evaluate the cytotoxic activity of anti-Gal IgM ABs in pre- and postoperative serum, α -Gal-bearing PK15-cells (a porcine renal cell line) were incubated with each serum. Cytotoxicity was increased in sera withdrawn 10 days after surgery, and could be diminished through pre-binding with soluble α -Gal (Fig.3.14. and 3.15.).

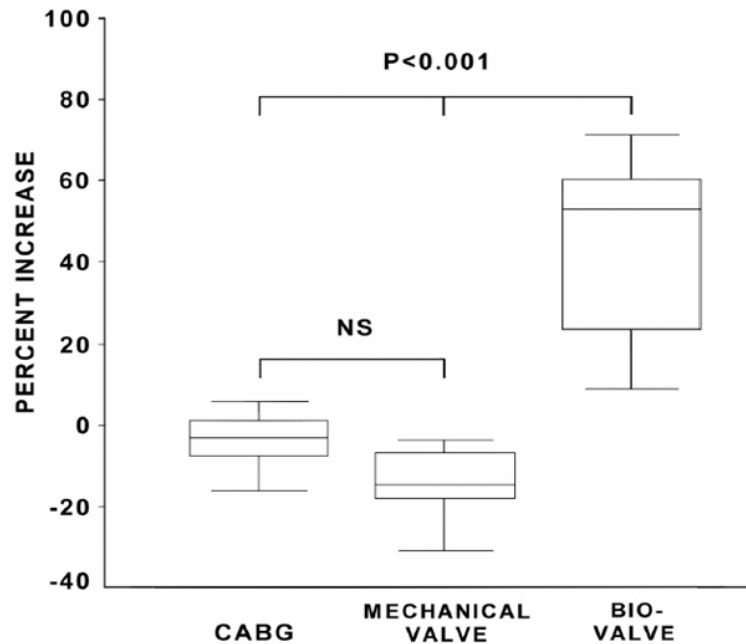


Figure 3.14: Recipients of bioprostheses demonstrating a significant increase of cytotoxic anti-galactose α 1,3-galactose IgM ABs as compared with control patients. Sera of recipients of bioprosthesis ($n=12$), mechanical bioprosthesis ($n=12$) and patients who underwent a CABG operation ($n=12$) were analyzed. Box plot shows the median, quartiles and extreme concentrations of percent increase of anti- α -Gal IgM ABs in serum ten days after the operation. A significantly increased mean \pm SEM at OD value 405 nm in the concentration of anti- α -Gal was observed in bioprosthesis valve recipients ($45.1 \pm 10.5\%$) as compared with recipients of mechanical prostheses ($-13.8 \pm 4.9\%$) and CABG patients ($-2.2 \pm 13.6\%$) (both, $p < 0.001$). Adapted from [2]

In conclusion, they described the presence of detectable α -Gal epitopes in GA-fixed bioprosthetic heart valves. Weigel *et al.* could confirm these results [141]. Furthermore, they detected an elicited IgM AB response after the implantation of biovalves and proved postoperative serum containing these ABs to be more potent in lysing viable porcine cells than preoperative serum of the same patient. They inferred that this antigen-AB connection could play a role in the degradation of bioprosthetic heart valves as these ABs opsonize Gal epitopes and initiate a specific Fc-receptor-mediated macrophage recruitment with antigen processing and antigen presentation.

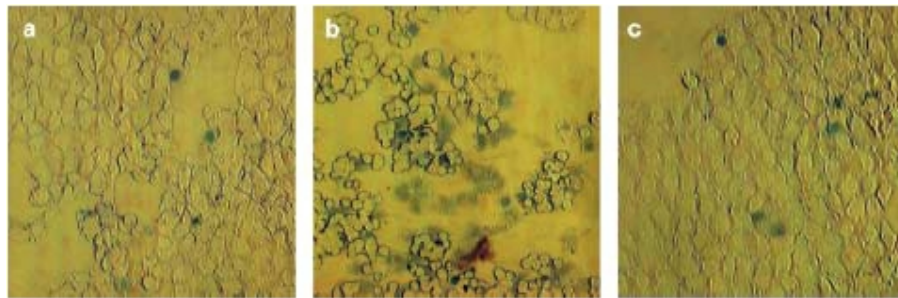


Figure 3.15: Photomicrograph. PK15 cells were grown on a glass-slide overnight and incubated with a serum pool diluted 1:8 from biovalve recipients ($n=10$) obtained preoperatively (a) and postoperatively (b). Pre-incubation of the postoperative serum pool with Gal α 1-3Gal α 1-4GlcNAc diminished its cytotoxic activity (c). Cells were stained with trypan blue and viewed with a Zeiss Axioplan 2 (Carl Zeiss, Jena, Germany). Adapted from [2]

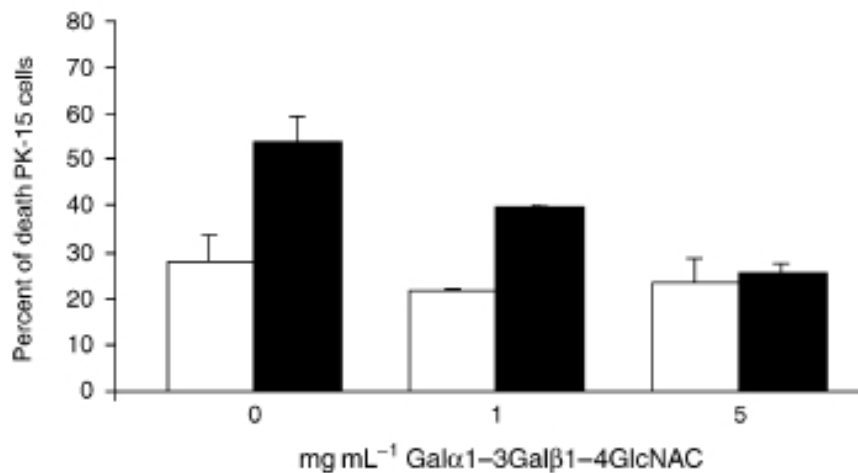


Figure 3.16: Cytotoxicity of anti galactose α 1,3-galactose ABs. PK15 cells were incubated with a serum pool diluted 1:4 from bioprostheses recipients ($n=10$) obtained preoperatively (white bars) and 10 days postoperatively (black bars). Necrosis was evaluated by staining with trypan blue. Error bars represent SD of two individual read-outs of the same experiment. One representative of three individually performed experiments is shown. The serum pool from postoperative sera induced a markedly higher necrosis in PK15 cells as compared with the sera obtained before surgery. Pre-incubation of serum pools with soluble inhibiting sugar Gal α 1-3Gal α 1-4GlcNAc abrogated cytotoxicity in a dose-dependent fashion. Adapted from [2]

3.4 Rationale and Aims

The destruction of bioprosthetic heart valves is a chronic process. Structural deterioration and calcification lead to dysfunction of the valve [79], and reoperation is necessary. In the study described above, our group was able to show that factors, which play an outstanding role in hyperacute rejection in xenotransplantation, are present after the implantation of bioprostheses. Obviously there is a humoral reaction to α -Gal epitopes in the valve tissue; certainly this reaction is restrained and not comparable to hyperacute rejection as evoked by viable xenograft organs. The question of the longevity of this humoral response has got to be raised.

In the present study we sought to investigate whether this humoral immune response is continuing and the augmentation of specific α -Gal IgM ABs results in an isotype-switch to IgG. Therefore, we planned to draw blood from valve recipients three months after valve implantation and measure α -Gal-specific IgG ABs compared to preoperative values. For further specification, we planned to define IgG subclasses as well.

Moreover, we planned to evaluate the presence of α -Gal in valve tissue of implanted bioprostheses after remaining one year in circulation. We used biovalves from patients who ceased one year after valve implantation and compared them to bioprostheses explanted after one week.

4 MATERIALS AND METHODS

4.1 Material

4.1.1 Reagents

Gal α 1.3–Gal β 1–4GlcNAc–R (Dextra Labobaroties, Reading, UK)

Goat Anti–Human IgG–Fc Polyclonal Antibody, Horseradish Peroxidase (HRP) Conjugated (Bethyl, Montgomery, USA)

Mouse Anti–Human IgG Monoclonal Antibody (all subclasses), HRP Conjugated (Invitrogen, Carlsbad, USA)

3,3',5,5'–Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma–Aldrich, St. Louis, USA)

Sulfuric acid 2N (H₂SO₄) (Sigma–Aldrich, St. Louis, USA)

Phosphat–buffered saline (PBS)-/- GIBCO™(Invitrogen, Carlsbad, USA)

Isolectin GS´–IB4 from Griffonia simplicifolia, Alexa Fluor 488 conjugate, Molecular Probes, Eugene, USA

VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Burlingame, USA
Hematoxylin (Sigma–Aldrich, St. Louis, USA)

Eosin (Sigma–Aldrich, St. Louis, USA)

4.1.2 Chemical Solutions

Washing buffer

PBS-/- GIBCO™

0.05% Tween 20 (Bio–Rad, Hercules, USA)

Carbohydrate buffer

Aqua dest. (BBraun, Melsungen, D)

Na₂CO₃ (Merck, Darmstadt, D)

NaHCO₃ (Merck, Darmstadt, D)

pH=9.6

Blocking buffer

PBS-/- GIBCO™

0.05% Tween 20

0.01% Bovine Serum Albumin (BSA) (Sigma–Aldrich, St. Louis, USA)

Sample diluent

0.05% Tween 20

1mM EDTA (Sigma–Aldrich, St. Louis, USA)

0.25% BSA

0.02% Thimerosal (Sigma–Aldrich, St. Louis, USA)

15mM Na₂B₄O₇ (Merck, Darmstadt, D)

120mM NaCl (Merck, Darmstadt, D)

pH=8.5

Fixing buffer

0.1M phosphate buffer (Merck, Darmstadt, D)

4% formaldehyde (Sigma–Aldrich, St. Louis, USA)

pH=7.4

Rinsing Solution for Histology

PBS-/- GIBCO™

5%, 10%, 20%, 30% Sucrose (Sigma–Aldrich, St. Louis, USA)

4.2 Methods

4.2.1 Human sera and clinical features

Human sera from patients who underwent valve replacement surgery (bioprosthetic valve replacement $n=19$, mechanical valve replacement $n=8$) were collected prior to, at ten days and at three months after surgery. The mean age of bioprosthetic and mechanical valve recipients was 74 ± 1.1 years and 56 ± 7.8 years, respectively. Since all mammals, except for Old World monkeys and humans express α -Gal on nucleated cells, we investigated recipients of both commercially available bovine and porcine bioprostheses in our clinical study. Types of bioprostheses implanted were: Sorin Pericarbon™ ($n=6$, Sorin S. p.A., Milano, Italy), Sorin Freedom™ ($n=7$), St. Jude Epic™ ($n=2$, St. Jude Medical, Inc., St. Paul, USA), Carpentier–Edwards Magna™ ($n=4$, Edwards Lifesciences, Irvine, USA). Three bioprosthetic tissue samples were obtained from patients who had to undergo re-operation because of valve malfunction or death within one week ($n=1$) and after 12–15 months ($n=2$), respectively, from the department of pathology, medical university vienna. This has been done in accordance with the ethics–committee, medical university vienna; a positive vote is present.

4.2.2 Enzyme–linked immunoabsorbent assays (ELISA)

ELISA technique was used to measure anti- α -Gal IgG and IgG subclass ABs in patients' sera prior to, on day ten days and at three months after cardiac surgery. The ELISA assay has been established by ourselves; extensive titration steps preceded definitive measurements. Gal α 1.3–Gal β 1–4GlcNAc–R was used as a solid–phase antigen. $10\mu\text{g}/\text{mL}$ were dissolved in carbohydrate buffer and coated on Nunc™96 well MaxiSorb™ plates overnight on 4° . Blocking was done with blocking buffer for two hours. After incubation with samples (two hours) and washing, the following HRP–conjugated detection ABs were added: anti–human IgG–Fc and anti–human IgG subclasses. After one hour and another washing step, color reaction was obtained with peroxidase reagent TMB and stopped with 2N sulfuric acid. Optical density was read at 450nm using a Victor3 plate reader (1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA). As no commercially human anti–Gal

AB is available, no standard concentration for absolute quantification could be performed. We used a dilution series of a reference serum from a healthy donor for intercomparability of measurements. Results were expressed as percentage increase/decrease and preoperative values were set as 100%.

4.2.3 Valve histology and immunohistochemistry

The explanted bioprostheses (after one week (n=2), after one year (n=3)) were fixed with 4% formaldehyde in 0.1M phosphate buffer (pH 7.4). Leaflets were rinsed in solutions of sucrose in PBS (5%, 10%, 20% and 30%) and then frozen in liquid nitrogen. 10µm sections were cut on a cryostat microtome (Kryocut model 3000; Leitz, Wetzlar, Germany) and mounted on gelatin-coated slides. These slides were stained with hematoxylin and eosin (HE). Furthermore, sections were double fluorescence-labeled with IB4 against α -Gal residues [142] (Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 488 conjugate) and DAPI against DNA in order to stain for nucleated cells; sections of explanted valves were rinsed in PBS, IB4 (1:500) was applied for two hours at 37°C. After rinsing, the sections were mounted with mounting medium containing DAPI. Labeled sections were analyzed and photo-documented under a confocal laser-scanning microscope (CLSM 510, Carl Zeiss, Jena, Germany).

4.2.4 Statistical Analysis

Statistical comparison of AB levels between time points and groups was performed using SPSS software (SPSS for Windows Version 15; SPSS Inc., Chicago, USA). Mann-Whitney-U test and Wilcoxon-test was used to calculate significance and a p-value<0.05 was considered to be statistically significant.

5 RESULTS

5.1 Evaluation of alpha–Gal specific Antibodies

5.1.1 Alpha–Gal specific IgG antibodies are significantly elevated three months after implantation of bioprosthetic heart valves

To test the hypothesis whether the implantation of bioprostheses increases α –Gal specific IgG, we used ELISA technique. Fig. 5.1. shows that α –Gal specific IgG ABs are significantly increased three months after valve replacement surgery compared to preoperative values (+21.7%, SEM \pm 4.65, $p < 0.001$) and to the control group ($p < 0.05$). No relevant difference between porcine and bovine bioprostheses could be observed. Recipients of mechanical heart valves do not show any elevation (-0.53%, SEM \pm 6.65, $p = 1.0$, NS [not significant]; Fig. 5.1.). The moderate decline of AB levels in both groups after ten days may be due to the postoperative state. However, no significance could be detected.

5.1.2 Three months after valve replacement, the specific IgG3 immune response is pivotal in recipients of bioprostheses

Since the implantation of bioprostheses increased total α –Gal specific IgG ABs, we extended our investigation to characterize IgG subclasses. Through that specification it will be possible to further narrow down impact of this finding, as IgG subclasses have got different affinities to receptors and humoral factors such as complement. As seen in Fig. 5.2., IgG3 increased significantly compared to preoperative values (+20.9%, SEM \pm 8.9, $p < 0.05$), and to the control group ($p < 0.01$, data not shown). The values for IgG1 were slightly increased as well. However, this did not reach significance.

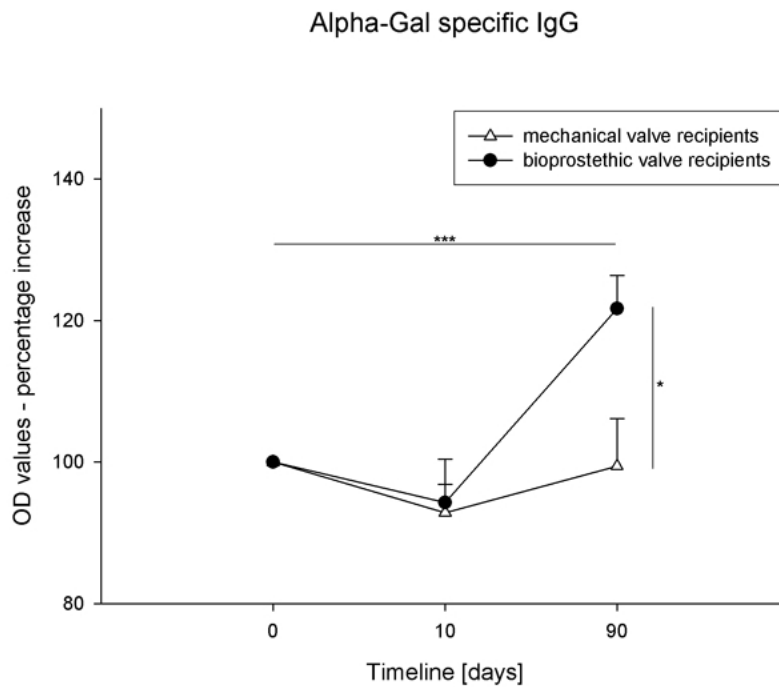


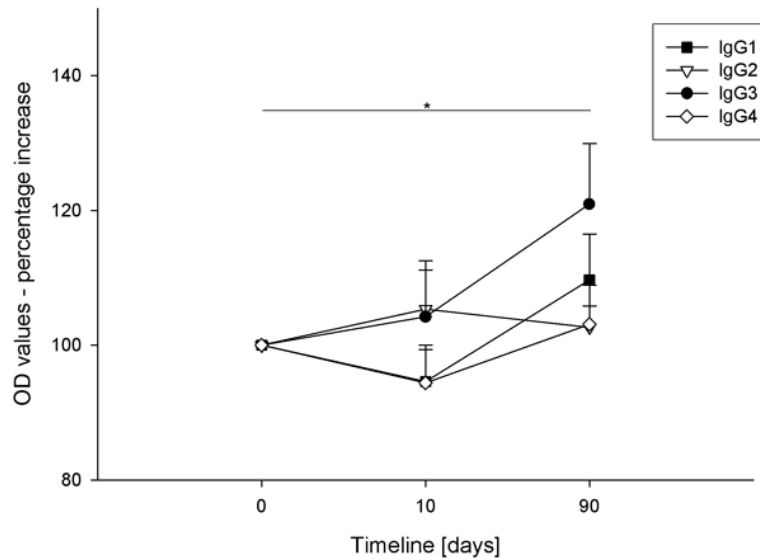
Figure 5.1: Evaluation of α -Gal specific IgG ABs revealed a significant increase three months after bio valve implantation ($***p < 0.001$) compared to preoperative values and compared to a control group ($*p < 0.05$), evidencing a specific long-lasting humoral immune response against the α -Gal epitope.

5.2 Determination of alpha-Gal presence on explanted bioprostheses

5.2.1 Time-dependent disintegration of IB4-positive structures in vivo on bioprosthetic heart valves

Fig. 5.3. and 5.4. show HE and CLSM images of an implanted bioprosthesis we received from the Department of Pathology (Medical University of Vienna) after remaining one week in circulation. In Fig. 5.3. (HE staining), a regular valve architecture and smooth tissue can be observed. Fig. 5.4. (CLSM) displays several DAPI-positive structures (blue, staining for DNA), and associated formations positive for IB4 (green, specific for α -Gal). This is concordant with previous results [2]. Fig. 5.5., 5.6. and 5.7. represent tissue

Alpha-Gal specific IgG subclasses - bioprosthetic valves



*Figure 5.2: The IgG subclass specification is depicted. Interestingly, IgG3 subclass levels are most affected (significant increase of IgG3, $*p < 0.05$, significantly higher than in the control group, $p < 0.01$, data not shown), whereas the other subclasses hardly respond to α -Gal structures of the implanted valve tissue.*

samples of bioprostheses explanted after 12 months because of malfunction. HE staining (Fig. 5.5.) reveals progressive destruction of the valve tissue; the collagen matrix appears much more condensed and fragmented than in Fig. 5.3. In the CLSM pictures (Fig. 5.6. and 5.7.) only background staining is visible, neither specific DAPI- nor IB4 staining can be detected.

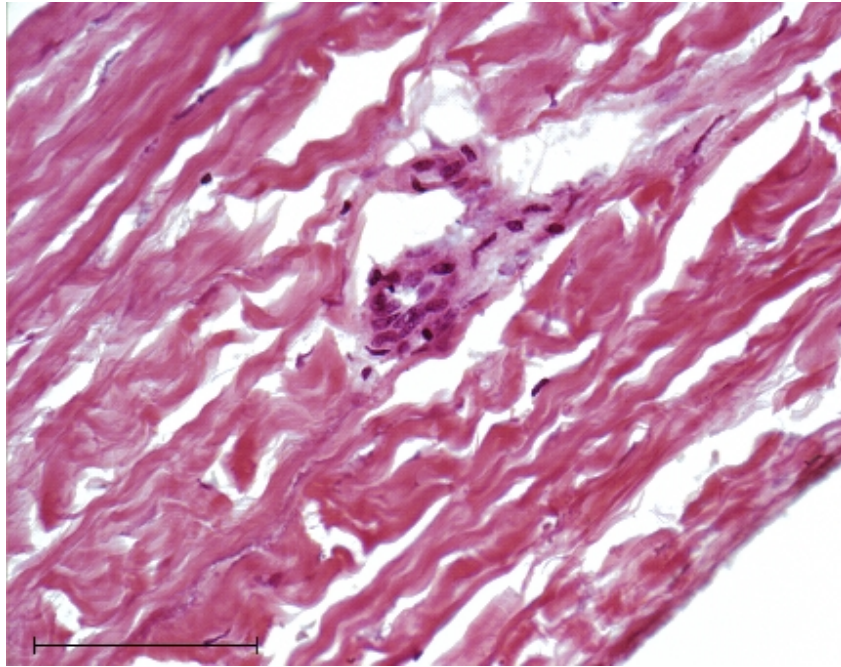


Figure 5.3: A porcine bioprosthesis explanted after 1 week: HE staining (light microscopy), scale bar: 10 μ m.

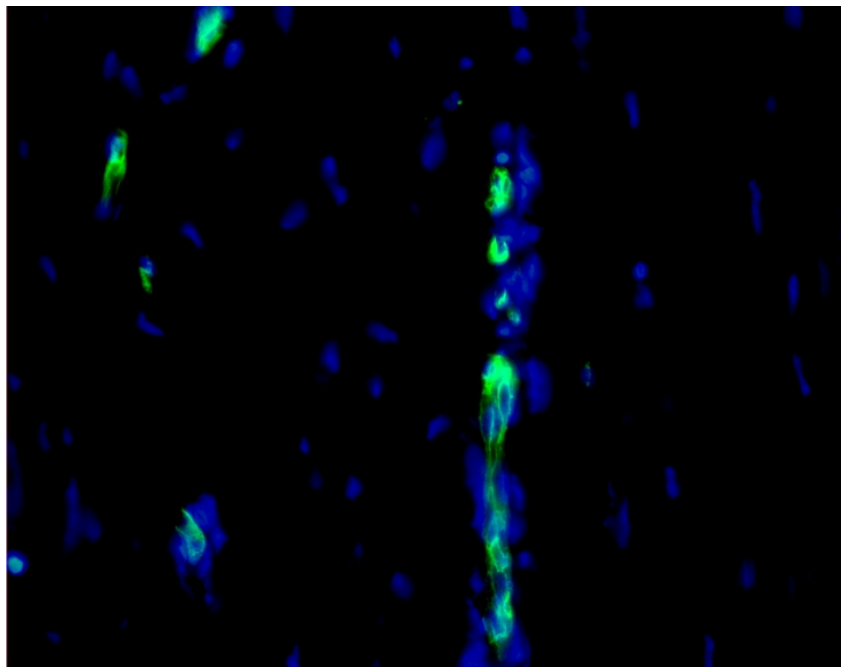


Figure 5.4: DAPI labeling shows numerous cell nuclei, many of those cells bearing IB₄-positive structures (α -Gal residues), scale bar: 100 μ m.

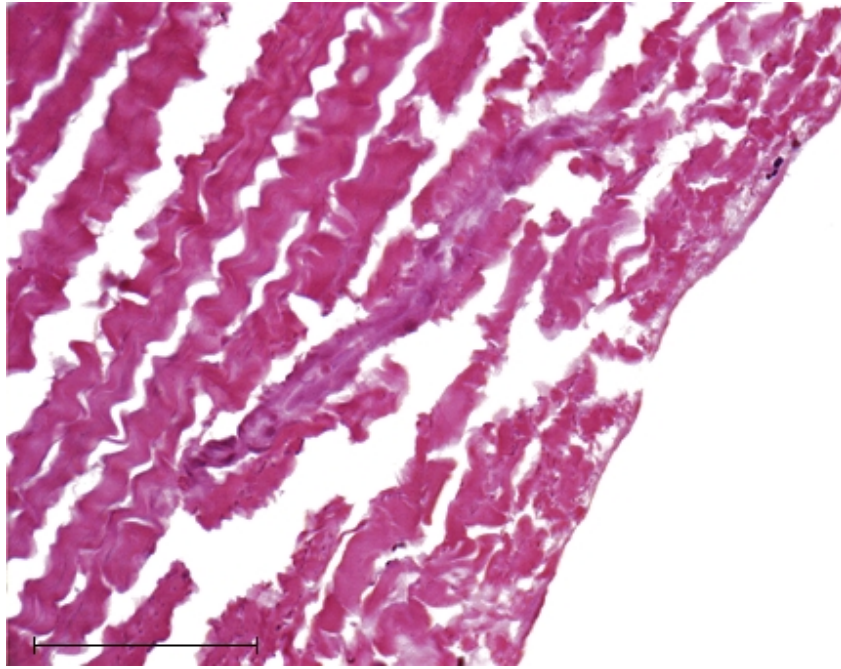


Figure 5.5: Porcine bioprosthesis explanted after 12 months. HE staining (light microscopy) shows progressive destruction of the valve matrix, scale bar: 10 μ m.

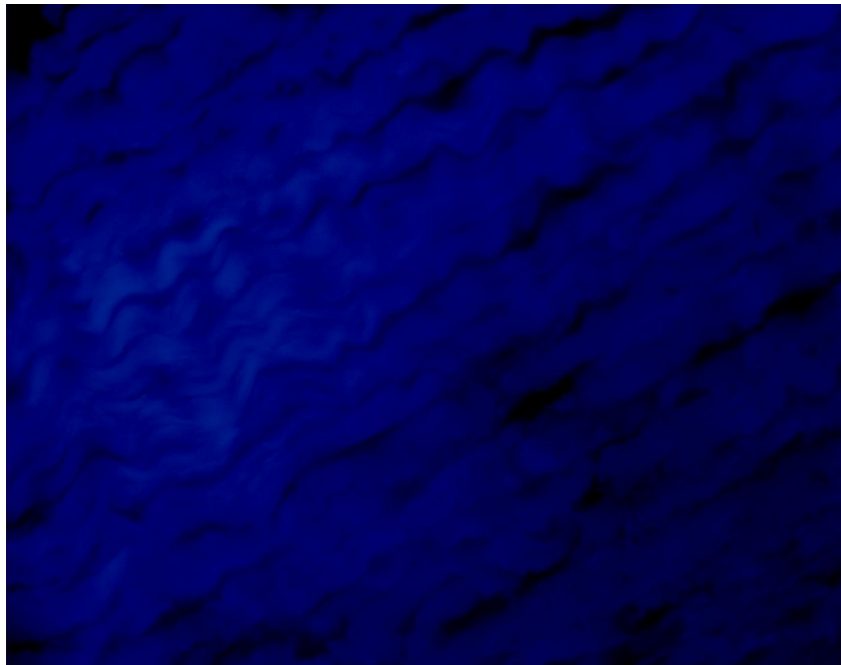


Figure 5.6: This image (CLSM) demonstrates total lack of cell nuclei (DAPI); only background staining is viewed.

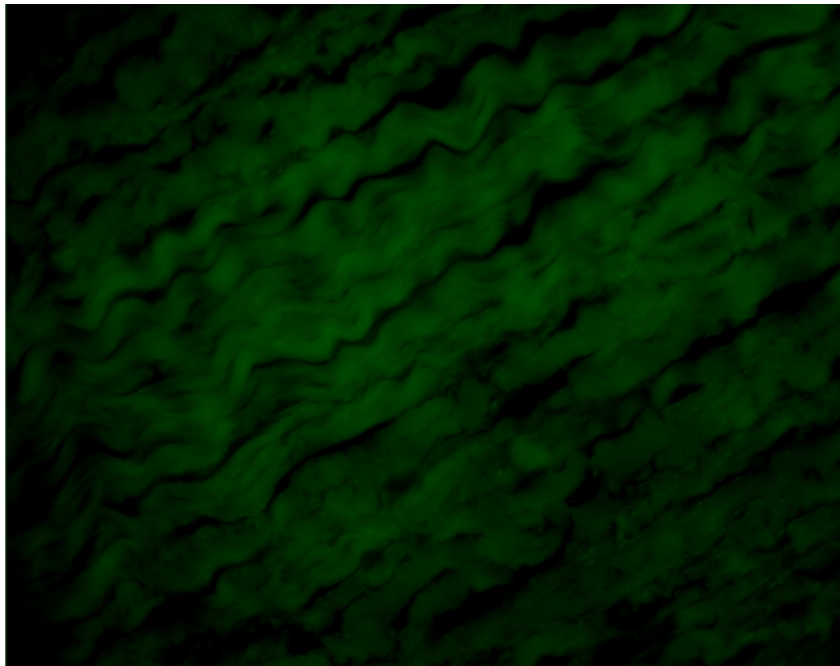


Figure 5.7: Consecutively, total absence of IB₄ positive structures, indicating that the α -Gal bearing cells present in valve tissue (see Fig.5.4.) are effectively degraded one year after implantation, scale bar: 100 μ m.

6 DISCUSSION

6.1 Discussion of Results

With the data presented here, we are able to extend our former results to formulate a hypothesis concerning an ongoing specific immune response against α -Gal on bioprosthetic heart valves. After valve implantation, α -Gal residues, which are present in the valve matrix, stimulate AB production, resulting in a significant increase of α -Gal specific IgM ABs ten days after operation [2].

We analyzed patients' sera at three months after bioprosthesis implantation and discovered an increase of α -Gal specific IgG ABs (Fig.5.1.). This indicates a common AB class switch induced by continuous antigen exposure. IgM+IgD+ B cells switch to expressing a different heavy chain, which causes another AB isotype to be synthesized. The light chains do not switch, hence the antigen specificity remains unaltered. This change of AB class is carried out through deletional DNA recombination. The switch may be driven to different isotypes, depending on several factors: (a) the type of antigen, (b) co-signaling via CD40 and (c) the cytokine constellation [143].

Our finding is essential, as it describes a prolonged activation of the immune system and provides an indication for the nature of this immune activation. The role of T cells in this scenario cannot be definitively determined. Potentially, T cell-dependent and -independent responses are possible. A T cell-independent response can be favored, based on the experiments of *Schoen and Levy* described above and the results reviewed by *Mond et al.* The former showed that T cells remain unaffected by the implantation of GA-fixed porcine tissue in rats [67, 68]. *Mond et al.* reviewed very well that polysaccharide antigens are mostly recognized without T cell support [144].

Our presented data revealed that IgG3 seems to be the major subclass isotype induced against α -Gal epitopes three months after valve implantation compared to control (Fig.5.2.). This was a surprise to us, as other studies describe mostly IgG2 as the predominant subclass induced against the Gal epitope [121, 145, 146]. A possible explanation could be as follows: In homeostasis, circulating anti-Gal IgG ABs mainly consist of the IgG2 subclass. These subclass IgG2 ABs activate complement poorly, and they may compete with complement-activating IgM ABs for antigen epitopes on e.g. bacte-

ria of the normal gut flora, as *Yu et al.* have speculated [147]. If novel Gal epitopes in humans occur, such as foreign material (xenograft heart valves), other co-factors and/or cytokines are released, and the isotype switch is developed towards IgG3, which is much more competent to activate humoral and cellular effects.

This kind of subclass switch to a more potent AB type is commonly known in memory cell-driven immune reactions [148]. Previously, it was shown in xenotransplantation that IgG3 is the major AB responsible for hyperacute xenograft rejection [149]. IgG3 is the IgG subclass known to be most capable of activating complement (C1q), especially at low antigen concentrations, and the most effective in inducing cytolysis [150, 151]. Its increasing predominance, particularly in response to decreasing antigen concentrations would be in perfect accord with the scenario taking place at a GA-fixed xenograft site, as described by our results [152]. Of utmost importance is the note that IgG3 is a powerful ligand of Fc-receptors: human neutrophils FcRIII-receptor (CD16) binds IgG3 complexes three times faster than other IgG subclass complexes. IgG3 is the most specific ligand known for low affinity Fc γ IIa (CD32) on monocytes, neutrophils and platelets [153, 154, 155].

It is reasonable to question why the anti-Gal AB titers should change so dramatically, although they are stimulated constantly by the gut flora, and are present at such extensive concentrations. This has been already discussed by *Yu et al.* [147], who measured anti-Gal titers after porcine liver perfusion for the treatment of acute hepatic failure and found a remarkable increase of anti-Gal-ABs in this condition: first, dosage of the antigen stimulus is heightened. How far this could play a role in the bioprostheses scenario, cannot be answered, but we consider it doubtful. More important seems to be the direct contact of the α -Gal antigen in blood perfusion with cells and humoral factors, and the altered presentation with different co-signals.

In our former work, we detected α -Gal structures in the subendothelial tissue of fixed, unfixed and commercially available porcine heart valves [2]. These results were corroborated by *Kasimir et al.* a few months later, another research group in Vienna [141]. This group examined commercially available porcine bioprostheses and porcine valve conduits with different decellularization protocols for the presence of α -Gal epitopes using CLSM. They

showed that bioprostheses and even decellularized valves containing intramural α -Gal.

Based on our previous work, we sought to investigate whether subendothelial α -Gal within the valve matrix is potent enough to launch a long-lasting immune response. Therefore, we designed this present study and detected a substantial augmentation of anti- α -Gal IgG, particularly IgG3. In addition, we performed immunohistochemical evaluation of explanted bioprostheses (explanted at a mean of one week and one year, respectively), scanning especially for cell nuclei and α -Gal residues. The H&E staining of biovalves that remained in circulation for one year shows extensive condensation and fragmentation (Fig.5.5.). If we interpret our results correctly, the following clinical scenario can be hypothesized: This condensation and fragmentation is the result of degradation and cleavage by MMPs and removal of cells out of the valve tissue. Bioprostheses explanted after one week reveal numerous cell nuclei with associated α -Gal residues within the valve matrix (Fig.5.4.), comparable to our former results (Fig.3.12.). In contrast, bioprostheses explanted after 12–15 months show neither detectable cell nuclei nor any α -Gal structures (Fig.5.6. and 5.7.). We speculate that the α -Gal bearing cells have been degraded by a consecutive immune reaction following opsonization through α -Gal specific ABs as described earlier.

In relation to previously published reports concerning α -Gal and xenograft valves, publications from the group around *Adams* have to be discussed. In 2000, *Chen et al.* reported the absence of α -Gal expression on unfixed porcine valves. Moreover, they did not find any sign of acute rejection after implanting unfixed porcine cardiac valves. *Chen* observed the time frame for hyperacute rejection, and investigated α -Gal structures only on the endothelial surface of unfixed porcine valves [156, 157]. In a following study in 2003, they revised their former assertion about the absence of Gal epitopes in porcine valve endothelial cells, as they found α -Gal expression using reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometry, albeit to a much lesser extent than in aortic or vein endothelial cells. In view of that, they admitted that delayed rejection of fresh porcine valves may occur [158].

In our previous work we produced ample evidence that numerous α -Gal-positive structures joint to nucleated cells are located within the tissue of unfixed, GA-fixed and commercially available bioprostheses. In accordance to *Chen et al.*, we did not detect any α -Gal on endothelium of fixed and unfixed valves by utilizing immunohistochemistry. Furthermore, we detected a specific immune response to these epitopes in human valve recipients which was clearly associated to valve implantation. Regarding (a) the impact of research in humans themselves compared to animal models, (b) the straightforward approach of our study and (c) the distinct results we were able to produce, make primate studies, such as those conducted by *Adams* group, questionable from our point of view.

Nevertheless, primate studies or studies in α -Gal-KO animals will be necessary, if calcification wants to be investigated adequately. We are confident that our findings have great impact on the topic of methodology, as we clearly show that the α -Gal barrier must be factored in xenograft valve destruction.

We can emphasize critical comments already raised by others [79] referring to the practice of implanting xenograft tissue subcutaneously in order to determine rejection and/or calcification. The physiological environment is obviously simply different and cannot be compared to areas within blood stream, where such tissue is exposed to humoral and cellular factors manifold. An even more substantial consequence of our findings affects the choice of model utilized to investigate xenograft degradation. Xenotransplantation models for rejection/calcification research within Gal-positive species are inappropriate. This must be explained in all clarity: If tissue is obtained from an α -Gal-positive species and implanted into another, likewise α -Gal-positive and therefore lacking anti-Gal ABs, the α -Gal barrier will not be addressed. Knowing that, the discrepancy between animal studies cited by valve producers showing excellent long-term results [159, 160] and the actually mostly moderate performance in patients [63] appears comprehensible. An already mentioned example is the unfortunate implantation of decellularized valves in pediatric patients, who experienced severe inflammation and destruction of their conduits soon [32], although studies in sheep (which are Gal-positive) had been very promising [161]. *Kasimir et al.* studied these results intensively and described the pathology taking place in these valves in great detail [162, 163]. In view of that, it is absolutely inexplicable why decellularized valves are still under testing in sheep to date [164, 165].

Another important contribution in the field of bioprosthesis valve research has been provided by *Manji et al.* in 2006 [88]. They reported animal experiments transplanting heart valves syn- and xenogenic between rats and guinea pigs. GA-fixed xenograft valves led to >3 times more valve inflammation, >10 times more valve T cell/macrophage infiltrate, and >3 times unspecific AB rise compared to fresh syngenic or the GA-fixed syngeneic groups. These results are a strong argument for the importance of the immune system in valve destruction. It must be emphasized that these valves have been transplanted in the infrarenal aorta position, and so are exposed to continuous blood flow. As explained above, valves are often implanted subcutaneously, which obviously represents pathological processes in the human blood circuit insufficiently. The author of an editorial comment deduced from these results that processing of allografts should strip off the endothelium, leading to a minimal number of cells being exposed to the blood stream and therefore evoking no humoral or cellular rejection response [166]. In view of our results, showing that α -Gal epitopes within the valve matrix are able to augment specific AB titers considerably, this theory is not convincing. Furthermore, in this editorial comment, the problem of α -Gal in xenograft heart valves was discussed. However, they fail to point out that just this major obstacle in animal-to-human graft donation cannot be tested in *Manji's* experimental series. Thus, the transferability of these results to the human system remains undetermined.

Recently, a study presented by *Lila et al.* [167] evaluated the calcification tendency of α -Gal-containing porcine pericardium tissue compared to pericardium from α -Gal-KO pigs. Since our data presumably provided the basis for this work, it is a pity that this publication is another example for questionable model choice. In detail, they implanted GA-fixed pericardial tissue, GA-fixed + formaldehyde, ethanol and Tween 80 (FET)-treated tissue, with and without pre-incubation with human anti-Gal ABs, subcutaneously into wistar rats during one month. They found a significant reduction of calcification of Gal-KO pericardium compared to wild-type tissue. Furthermore, calcification was greatly reduced through addition of FET. When the implants were pre-incubated with human anti-Gal ABs, Gal-positive tissue revealed to have strongly enhanced calcification compared to Gal-negative tissue, or any tissue not pre-incubated with such ABs. The methodological concept of that work is not comprehensible. (a) The tissue samples were implanted subcutaneously. The authors suggest, contrary to current knowledge, that this method is adequate as it is traditionally used and empirically well validated. This

argument is not convincing to us as discussed above. (b) As correctly reviewed by *Lila*, humans and old world monkeys are the only mammals who lack the α -Gal epitope but display high anti-Gal titers. Thus, rats are the wrong model, if the immunological role of α -Gal wants to be addressed. Rats express the Gal epitope themselves, and more importantly have no systemic anti-Gal ABs in consequence. Therefore, an α -Gal-specific immune response to α -Gal cannot occur. (c) However, we concede the pre-incubation with human anti-Gal ABs is an approach towards an immunological linkage; but the implicated presumption of the authors, that ABs can accomplish effector functions in a xenogenic system such as human ABs in a rat organism, is unsustainable. This may be possible, but the actual scenario taking place can never be defined clearly, and has not even been tried in the present study. So far, the presented results are lacking explanation. Nevertheless, they are at hand; it would be restrictive not to consider them. We know that Gal epitopes and the respective ABs can differ slightly amongst each other [168], this may could be a possible explanation favoring an immunological background. However, this is unlikely, from our point of view, and would have to be proven. As immunological reasons for the significant difference in calcification of α -Gal-negative tissue must be questioned, other possible explanations should be evaluated.

6.2 Conclusion

6.2.1 Clinical Implications

The 2008 update of the ACC/AHA guidelines for the management of patients with valvular heart disease recommends the use of bioprosthetic heart valves for patients over 65 and in patients under 65 who are unwilling to accept anticoagulative therapy. The choice of valve for women of childbearing age is difficult. Surgeons tend to recommend biological valves since mechanical valve recipients require marcoumar therapy.

Other factors that should be considered: (a) Overall, the general trend tends towards biological xenograft valves. Anticoagulation is an unpopular therapy amongst patients and surgeons because of the risk of bleeding and quality of life. The number of reoperations will increase due to better medical care and lowering of operability limitations. This fur-

ther increases costs and morbidity. (b) Novel aortic valve replacement via a transapical and transfemoral route has been advocated by a number of prominent centers. For this heavily promoted therapy, bioprostheses are the only available choice due to the pliability of the valve [169, 170]. (c) Atrial fibrillation (AF) is a common disease in the older patient. Up to 50% of the patients receiving cardiac surgery experience paroxysmal AF with the risk of persistence [171]. People over 70 have an incidence of 10% suffering from chronic AF [172], and because of that, this patient cohort needs anticoagulation anyway. This should give rise to more encouragement for extended research towards a better understanding of the pathogenesis of xenograft valve degeneration. Since we show in this work that an α -Gal specific humoral immune response is induced after implantation of bioprostheses, we recommend caution with regard to an uncritical lowering of age limits (<65 yrs) for implantations in patients with valve disease.

For all the above reasons, bioprostheses need to be improved. As long as better long-term performance cannot be achieved, current ACC/AHA guidelines should be strictly followed. In conclusion, we are confident that our results will lead to an enhanced caution towards the commonly aired opinion which suggests that an immune response in bioprostheses recipients is of no clinical relevance.

6.2.2 Outlook

To the best of our knowledge, this is the first description of a long-lasting specific humoral response to commercially available xenograft (porcine, bovine and equine) bioprostheses. Whether this humoral activation is followed by pathophysiological processes including cell migration and activation, complement and cytokine release resulting in valve degeneration, remains an object of further research. Only appropriate animal models, regarding the α -Gal epitope and anti-Gal ABs, will lead to reliable data concerning xenograft valve calcification and degradation. Ultimately, the explanatory connection between immune response and valve calcification will have to be drawn to determine the actual importance of such a response in the pathogenesis of the failing bioprosthetic heart valve.

7 ABBREVIATIONS

α -Gal	Gal α 1.3-Gal β 1-4GlcNAc-R
α 1,3GT	β -galactosyl α 1-3-galactosyltransferase
AB	Antibody
ADCC	antibody dependent cellular cytotoxicity
AF	atrial fibrillation
APC	antigen-presenting cells
AR	aortic regurgitation
AS	aortic stenosis
AV	aortic valve
AVR	aortic valve replacement
BSA	bovine serum albumine
CABG	coronary artery bypass grafting
CLSM	confocal laser scan microscopy
DNA	desoxyribonucleic acid
ELISA	enzyme-linked immunoabsorbent assay
FET	formaldehyde, ethanol and Tween 80
GA	glutaraldehyde
hDAF	human decay accelerating factor
HE	hematoxylin and eosin
HRP	horseradish peroxidase
IgM,E,A,D,G	Immunoglobulin M,E,A,D,G
KO	knockout
LV	left ventricle
LVOT	left ventricular outflow tract
MAC	membrane attack complex
MMP	matrix-metalloproteinase
MR	mitral regurgitation
MS	mitral stenosis
MV	mitral valve
NK cells	natural killer cells
NYHA	new york heart association
oxLDL	low-density lipoprotein
PBS	phosphate buffered saline
PERV	porcine endogenous retrovirus
RT-PCR	reverse transcriptase – polymerase chain reaction
TMB	3,3',5,5'-Tetramethylbenzidine
VR	valve replacement

Table 7.1: Abbreviations

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11 APPENDIX A

11.1 Curriculum vitae

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-
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11.2 Research Activity and Publications

11.2.1 Articles

Mangold A, Ankersmit HJ. Letter to the editor concerning the recently published paper by Lila et al. *J Heart Lung Transplant*. 2010; *ahead of print*

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11.2.3 Poster Presentations

Mangold A, Hoetzenecker K, Hacker S, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal specific humoral immune response after implantation of bioprostheses in cardiac surgery. *Austrotransplant 2007, St. Wolfgang; OEGAI 2007, Alpbach*

12 APPENDIX B

Alpha-Gal Specific IgG Immune Response after Implantation of Bioprostheses

Authors A. Mangold¹, T. Szerafin², K. Hoetzenecker¹, S. Hacker¹, M. Lichtenauer¹, T. Niederpold¹, S. Nickl¹, M. Dworschak², R. Blumer², J. Auer², H. J. Ankersmit¹

Affiliations The affiliations are listed at the end of the article

Key words

- heart valve surgery
- bioprosthesis heart valves
- mechanical heart valves
- xenograft
- alpha-Gal

Abstract



Background: We have previously shown that the α -Gal (Gal α 1.3-Gal β 1-4GlcNAc-R) epitope is a relevant xenoantigen present on bioprostheses utilized in cardiac surgery and elicits an α -Gal specific IgM immune response. We sought to investigate whether that immune response continues after valve implantation.

Materials and Methods: We collected plasma samples from patients who underwent bioprosthesis implantation (n = 19) or mechanical valve replacement (n = 8), respectively, prior to, at 10 days and at 3 months after cardiac surgery. ELISA was utilized to quantify α -Gal specific IgG and IgG subclasses. 3 bioprosthetic tissue samples were obtained from patients who had to undergo reoperation within 1 week (n = 1) or at 12–15 months (n = 2) after the initial operation. We utilized confocal laser scanning microscopy (CLSM) to detect the presence of α -Gal epitopes (IB4) and cell nuclei (DAPI).

Results: α -Gal specific IgG was significantly increased 3 months after implantation of bioprostheses compared to preoperative values ($p < 0.001$) and was significantly higher than α -Gal specific IgG levels of the control group ($p < 0.05$). IgG3 was the major subclass directed against α -Gal ($p < 0.05$, pre- vs. postoperative values). In CLSM analysis we demonstrated that bioprostheses explanted 1 week after implantation contained IB4/DAPI positive cells within the collagen matrix. In contrast, in patients who underwent reoperation after 12 months, porcine tissue showed a complete lack of IB4/DAPI.

Conclusion: Our results indicate that the implantation of bioprostheses elicits a specific humoral immune response against α -Gal bearing cells compared to controls within 3 months after cardiac surgery. The complete absence of IB4/DAPI positive structures 12 months after implantation indicates a specific degradation of α -Gal bearing cells through previous exposure to the human blood circuit.

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Introduction



α -Gal (Gal α 1.3-Gal β 1-4GlcNAc-R) is a carbohydrate epitope present on the cells of all mammals except for humans and Old World monkeys [1]. Humans customarily display high amounts (about 1%, [2]) of natural anti-Gal antibodies [3,4] at about 3 months after birth due to antigenic stimulation through α -Gal-expressing enterobacteria of the developing gut flora, similar to ABO antibodies [5,6]. Consequently, α -Gal is a major antigen in hyperacute xenograft rejection [7]. Bioprosthetic heart valves are widely utilized in valve replacement surgery because, in contrast to mechanical heart valves, no lifelong anticoagulation is needed [8]. However, the major disadvantage is the limited durability due to tissue degeneration and calcification. Moreover it was shown

that young patients have an increasing tendency to calcification due to immunological processes [8–10]. Although a lot of research is being done to increase the understanding of the pathophysiology of the degeneration process, the discussion regarding the major causes remains ongoing [11–13].

In our previous work, we demonstrated that α -Gal is still present in commercially available glutaraldehyde-fixed porcine and bovine bioprosthetic heart valves. We were able to show (a) collagen tissue of bioprostheses with interspersed fibrocytes that stained positive with IB4, a lectin that binds specifically to alpha-D-galactosyl residues [14], indicating the presence of α -Gal sugar epitopes; and (b) that recipients of bioprostheses developed an augmentation of α -Gal specific IgM antibodies [15].

Mangold A et al. Alpha-Gal Specific IgG... Thorac Cardio Surg 2009; 57: 191–195

Figure 12.1: Publication in *The Thoracic and Cardiovascular Surgeon*, Page 1 [173]

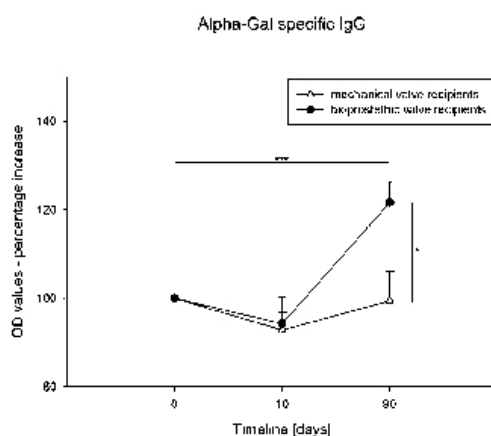


Fig. 1 Evaluation of α -Gal specific IgG revealed a significant increase 3 months after bio valve implantation (** $p < 0.001$) compared to preoperative values and compared to a control group (* $p < 0.05$), evidencing a specific humoral immune response against the α -Gal epitope.

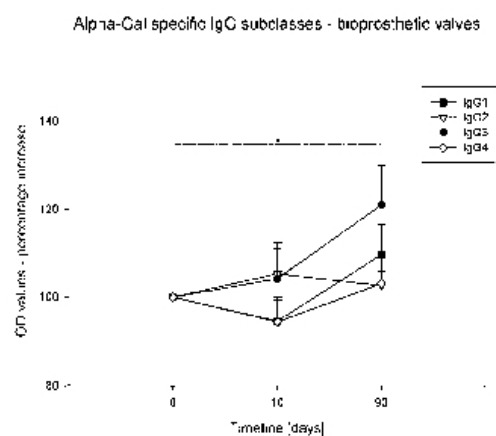


Fig. 2 The IgG subclass specification is depicted in **Fig. 2**. Interestingly, IgG3 subclass levels are most affected (significant increase of IgG3, * $p < 0.05$, significantly higher than in the control group, $p < 0.01$, data not shown), whereas the other subclasses hardly respond to α -Gal structures of the implanted valve tissue.

Due to this observation of an α -Gal specific humoral immune response after bio valve implantation, we hypothesized whether α -Gal specific IgG content increases within 3 months after valve implantation. Furthermore, we explored which α -Gal specific IgG subclass is increased within the same period. Lastly, we examined bioprostheses explanted after 1 week and after 12 months, respectively, to investigate eventual changes in IB4 staining.

Materials and Methods

Human sera and clinical features

Human sera from patients who underwent valve replacement surgery (bioprosthetic valve replacement $n = 19$, mechanical valve replacement $n = 8$) were collected prior to, at 10 days and at 3 months after surgery. The mean age of bioprosthetic and mechanical valve recipients was 74 ± 1.1 years and 56 ± 7.8 years, respectively. Since all mammals, except Old World monkeys and humans express α -Gal on nucleated cells, we investigated recipients of commercially available bovine and porcine bioprostheses in our clinical study. Types of bioprostheses implanted were: Sorin Pericarbon™ ($n = 6$, Sorin S. p. A., Milano, Italy), Sorin Freedom™ ($n = 7$), St. Jude Epic™ ($n = 2$, St. Jude Medical, Inc., St. Paul, MN, USA), Carpentier-Edwards Magna™ ($n = 4$, Edwards Lifesciences, Irvine, CA, USA). 3 bioprosthetic tissue samples were obtained from patients who had to undergo reoperation because of valve malfunction or death within one week ($n = 1$) and after 12–15 months ($n = 2$), respectively.

Enzyme-linked immunoabsorbent assays

The ELISA technique was used to measure anti- α -Gal IgG and IgG subclass antibodies in patients' sera prior to, at 10 days and at 3 months after cardiac surgery. Galalpha1.3-Gal β 1-4GlcNAc (Dextra Laboratories, Reading, UK) was used as a solid-phase antigen. Blocking was done with assay buffer (2.5 g BSA, 500 ml PBS $-/-$, 250 μ l Tween 20). After incubation with samples and washing,

the following HRP-conjugated detection antibodies were added: antihuman IgG-Fc (Bethyl, Montgomery, TX, USA) and antihuman IgG subclasses (Invitrogen, Carlsbad, CA, USA). A color reaction was obtained with peroxidase reagent TMB (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich, St. Louis, MO, USA) and optical density was read at 450 nm using a Victor3 plate reader (1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA).

Valve histology and immunohistochemistry

Histology and immunohistochemistry were performed as reported before [15]. In brief, the explanted bioprostheses were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Leaflets were rinsed in solutions of sucrose in PBS and then frozen in liquid nitrogen. 10- μ m section were cut on a cryostat microtome (Kryocut model 3000; Leitz, Wetzlar, Germany) and mounted on gelatin-coated slides. These slides were stained with hematoxylin and eosin (H&E).

Furthermore, sections were double fluorescence labeled with IB4 against α -Gal residues [14] (Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 488 conjugate, catalogue # 1-21411, Molecular Probes, Eugene, OR, USA) and DAPI (VECTASHIELD Mounting Medium with DAPI, catalogue # H-1200, Vector laboratories, Burlingame, CA, USA) against DNA in order to stain for nucleated cells; sections of explanted valves were rinsed in PBS, IB4 (1:500) was applied for 2 h at 37 °C. After rinsing the sections were mounted with mounting medium containing DAPI.

Labeled sections were analyzed and photo-documented under a confocal laser-scanning microscope (CLSM 510, Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS for Windows Version 15; SPSS Inc., Chicago, IL, USA). Mann-Whitney U test and Wilcoxon-test was used to calculate significance and a p value of 0.05 was considered to be statistically significant.

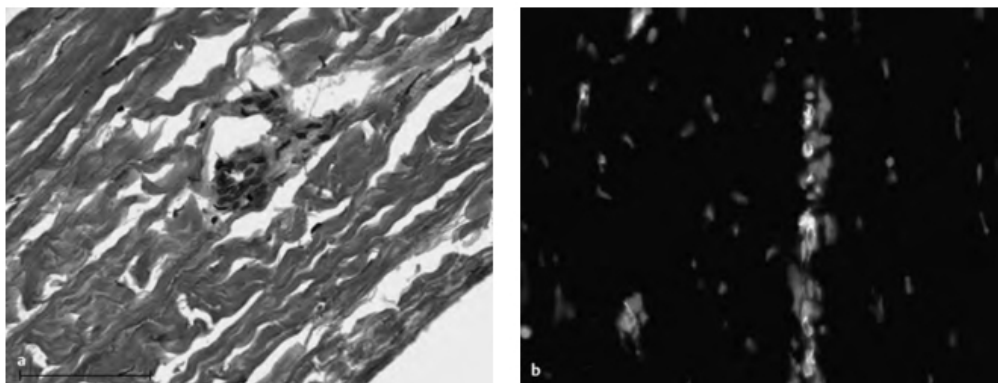


Fig. 3a and b A porcine bioprosthesis explanted after 1 week. **a** H&E staining (light microscopy), scale bar: 10 μ m. **b** IB4 (green) and DAPI (blue)

labeling (CLSM). DAPI labeling shows numerous cell nuclei, many of those cells bearing IB4-positive structures (α -Gal residues), scale bar: 100 μ m.

Results

α -Gal specific IgG antibodies are significantly elevated 3 months after implantation of bioprosthetic heart valves

To test the hypothesis whether the implantation of bioprostheses increases α -Gal specific IgG, we used the established ELISA technique. **Fig. 1** shows that α -Gal specific IgG antibodies are significantly increased 3 months after valve replacement surgery compared to preoperative values (+21.7%, SEM \pm 4.65, $p < 0.001$) and to the control group ($p < 0.05$). No relevant difference between porcine and bovine bioprostheses could be observed. Recipients of mechanical heart valves do not show any elevation (-0.53% , SEM \pm 6.65, $p = 1.0$, NS [not significant]; **Fig. 1**).

Three months after valve replacement surgery, the specific IgG3 immune response is pivotal in recipients of bioprostheses

Since the implantation of bioprostheses increased total α -Gal specific IgG immunoglobulins, we extended our investigation to characterize IgG subclasses. As seen in **Fig. 2**, IgG3 increased significantly compared to preoperative values (+20.9%, SEM \pm 8.9, $p < 0.05$), and to the control group ($p < 0.01$, data not shown). The values for IgG1 were slightly increased as well. However, this did not reach significance.

Time-dependent disintegration of IB4 positive structures *in vivo* on bioprosthetic heart valves

Fig. 3a, b show H&E and CLSM images of a bioprosthesis explanted after one week due to malfunction. In **Fig. 3a** (H&E staining), a regular valve architecture and smooth tissue can be observed. **Fig. 3b** (CLSM) displays several DAPI-positive structures (blue, staining for DNA), and associated formations positive for IB4 (green, specific for α -Gal). **Fig. 4a–c** represent tissue samples of bioprostheses explanted after 12 months because of malfunction. H&E staining (**Fig. 4a**) reveals progressive destruction of the valve tissue; the collagen matrix appears much more condensed and fragmented than in **Fig. 3a**. In the CLSM pictures (**Fig. 4b, c**) only background staining is visible, neither specific DAPI- nor IB4 staining can be detected.

Comment

Using the data presented here, we have been able to extend our former results to formulate a hypothesis concerning an ongoing specific immune response against α -Gal on bioprosthetic heart valves. After valve implantation, α -Gal residues, which are present in the valve matrix, stimulate antibody production, resulting in a significant increase of α -Gal specific IgM antibodies ten days after operation [15]. We analyzed patients' sera at 3 months after bioprosthesis implantation and found a similar increase of α -Gal specific IgG antibodies. This indicates a common antibody class switch induced by continuous antigen exposure [16]. Further specification revealed that IgG3 seems to be the major subclass isotype induced against the α -Gal epitopes. IgG3 is the IgG subclass known to be most capable of activating complement (C1q), especially at low antigen concentrations, and the most effective in inducing cytolysis [17–19]. Furthermore IgG3 is a powerful ligand of Fc γ -receptors: human neutrophils Fc γ RIII-receptor (CD16) binds IgG3 complexes 3 times faster than other IgG subclass complexes; IgG3 is the most specific ligand known for Fc γ II (CD32) [20–22].

The role of immunological processes in xenograft valve degeneration has been investigated for a long time [23,24], and recent convincing results have been reported [25]. Nevertheless, the discussion concerning the major causes remains ongoing.

Chen et al. reported in 2000 the absence of α -Gal expression on unfixed porcine valves [26]. Chen observed a time frame of hyperacute rejection, and investigated α -Gal structures only on the endothelial surface of unfixed porcine valves. In another study in 2003, the same group had to revise their assertion of 2000 because the results of reverse transcriptase-polymerase chain reaction and flow cytometry investigations revealed that there is α -Gal expression even in endothelial valve cells, albeit to a much lesser extent than in aortic or vein endothelial cells [27]. This coincides with findings in our former work, where we detected α -Gal structures in the subendothelial tissue of fixed and unfixed porcine heart valves. The same results were found by Kasimir et al. [28]. This group examined commercially available porcine bioprostheses and porcine valve conduits with different decellularization protocols for the presence of α -Gal epitopes using CLSM. Bioprostheses and even decellularized valves showed intramural

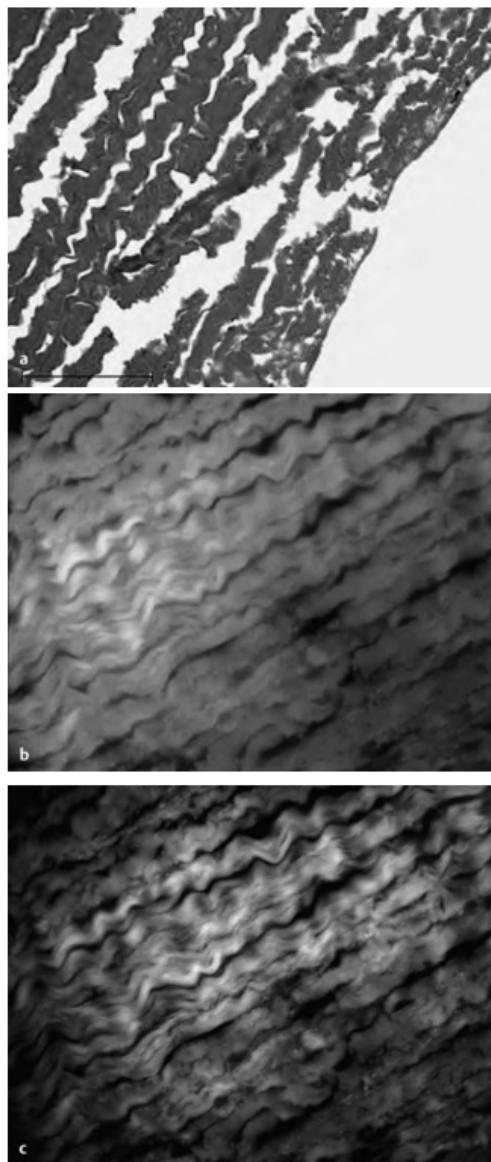


Fig. 4a to c Porcine bioprosthesis explanted after 12 months. H&E staining under **a** (light microscopy) shows progressive destruction of the valve matrix, scale bar: 10 μ m. **b, c** Images (CLSM) demonstrate a total lack of cell nuclei (DAPI, only background staining is seen) or IB4-positive structures, indicating that the α -Gal bearing cells present in valve tissue (s. **Fig. 3b**) are effectively degraded one year after implantation, scale bar: 100 μ m.

α -Gal. To evaluate whether subendothelial α -Gal is potent enough to launch a long-lasting immune response, we designed this present study and detected a substantial augmentation of IgG.

We furthermore scanned explanted bioprostheses for cell nuclei and α -Gal with CLSM. Bioprostheses explanted after one week revealed numerous cell nuclei with associated α -Gal residues within the valve matrix, whereas bioprostheses explanted after 12–15 months showed neither detectable cell nuclei nor any α -Gal structures. We speculate that the α -Gal bearing cells had been degraded by a consecutive immune reaction following opsonization through α -Gal specific antibodies.

Clinical implication and outlook

The 2008 update of the ACC/AHA guidelines for the management of patients with valvular heart disease recommends the use of bioprosthetic heart valves for patients over 65 and in patients under 65 who are unwilling to accept anticoagulative therapy. A similar advice is given for women of childbearing age [29]. Moreover, novel aortic valve replacement via a transapical and transfemoral route has been advocated by a number of prominent centers. For this heavily promoted therapy α -Gal carrying bioprostheses are the only available choice due to the pliability of the valve [30, 31]. Since we show in this work that an α -Gal specific humoral immune response is induced after implantation of bioprostheses, we recommend caution with regard to an uncritical lowering of age limits (< 65 yrs) for implantations in patients with valve disease [9]. We are confident that our results presented here will lead to an enhanced caution concerning the commonly aired opinion which suggests that immune response in bioprostheses recipients is of no clinical relevance.

Conclusion

To the best of our knowledge this is the first description of a long-lasting humoral response to routinely transplanted xenograft valves in literature. Whether this humoral activation is the sole responsible mechanism or other cellular components are involved in the valve degeneration process, as proposed by Human and Zilla [12], remains an object of further research.

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13 APPENDIX C

13.1 Letter to the Editor, accepted in *Journal of Heart and Lung Transplantation*

Comment on: Lila N, McGregor CG, Carpentier S, Rancic J, Byrne GW, Carpentier A. **Gal knockout pig pericardium: New source of material for heart valve bioprotheses.** *J Heart Lung Transplant.* 2009 ahead of print [167]

To the editor:

In the study presented recently by *Lila et al.* [167], they evaluated the calcification tendency of α -Gal-containing porcine pericardium tissue compared to pericardium from α -Gal-knockout (KO) pigs. As our data provided the basis for this work, we want to comment on it. In 2005, we described α -Gal epitopes in glutaraldehyde (GA)-fixed bioprosthetic heart valves for the first time and definitively disproved the often raised assertion that the fixation process of biological valves is abolishing antigenicity of the tissue. Moreover, we were able to detect a significant elevation of anti- α -Gal IgM antibodies in bioprotheses recipients ten days after operation [2]. Recently, we showed that α -Gal-specific IgG antibodies are significantly increased in patients' sera three months after bioprosthetic valve implantation. This is the proof for a long-lasting immune response to α -Gal residues within the valve tissue. In biovalves explanted after remaining one year in circulation, we neither detected α -Gal-positive structures nor any cell nuclei, indicating that these cells had been effectively degraded while being exposed to the blood circuit [173].

In detail, *Lila et al.* implanted GA-fixed tissue, GA-fixed + formaldehyde, ethanol and Tween 80 (FET)-treated tissue, with and without preincubation with human anti-Gal antibodies, subcutaneously into wistar rats during one month. They found a significant reduction of calcification of Gal-KO pericardium compared to wild-type tissue. Furthermore, calcification was greatly reduced through addition of FET. When the implants were pre-incubated with human anti-Gal antibodies, Gal-positive tissue revealed to have strongly enhanced calcification compared to Gal-negative tissue, or any tissue not pre-incubated with such antibodies. These interesting results need to be discussed in more detail. What puzzled us is the methodologic concept chosen in this work. (a) The tissue samples were implanted subcutaneously. The authors explain that this method is adequate as it is traditionally used and empirically well validated. This argument is not

convincing, as the environment is physiologically simply different. *Manji et al.* delivered distinct data for xenograft calcification by valve implantation in rats into the infrarenal aortic position, compared to former subcutaneous models [88]. (b) As correctly reviewed by *Lila*, humans and old world monkeys are the only mammals who lack the α -Gal epitope but display high anti-Gal titers. Thus, rats are the wrong model, if the immunological role of α -Gal wants to be addressed. Rats express the Gal epitope themselves, and do not have anti-Gal antibodies. Therefore, a specific immune response to α -Gal is hardly possible. (c) The preincubation with human anti-Gal antibodies is an approach towards an immunological linkage; but the implicated assumption of the authors, that antibodies can accomplish effector functions in a xenogenic system such as human antibodies in a rat organism, is unsustainable. This may be possible, but the actual scenario taking place can never be defined clearly, and hasn't even been tried in the present study. So far, the presented results are lacking explanation. Nevertheless, they are at hand; it would be restrictive not to consider them. We know that Gal epitopes and the respective antibodies can differ slightly amongst each other [168], this maybe could be a possible explanation favoring an immunological background. However, this is unlikely, from our point of view, and would have to be proven. As immunological reasons for the significant difference in calcification of α -Gal-negative tissue must be questioned, other possible explanations should be evaluated. We are content that our results are encouraging researchers to seek for deeper insights concerning α -Gal and its role in bioprosthetic heart valve destruction, but emerging obstacles shall be overcome rather than evaded.