# Reverse Remodeling of Cardiac Collagen Protein Expression after Surgical Therapy for Experimental Aortic Stenosis

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*Background and aim of the study:* **Reversal of myocardial collagen gene expression was examined in parallel to left ventricular reverse remodeling after surgical correction of experimental aortic stenosis.**
*Methods:* **A standard growing sheep model (age at baseline 6-8 months) was used. Measurements were performed at baseline (point A) when inducing left ventricular hypertrophy (LVH) through supracoronary banding, at 8.3 ± 1.0 months later at surgical correction (point B), and after another 10.1 ± 2.0 months during final examinations (point C). Gene expression for collagen I and III was also studied.**
*Results:* **Left ventricular function was stable throughout the study. The left ventricular mass index (LVMI) was 82 ± 21 g/m$^2$ at point A, 150 ± 33 g/m$^2$ at B, and 78 ± 18 g/m$^2$ at C ($p < 0.01$). Myocardial fiber diameter was 11.3 ± 0.8, 15.9 ± 1.2 and 11.4 ± 1 μm at points A, B and C, respectively ($p < 0.01$). Protein expression for collagen I was 0.71 ± 0.2 (at A), 1.13 ± 0.3 (at B) and 0.85 ± 0.4 (at C) ($p < 0.01$), while that for collagen III was 0.72 ± 0.4 (at A), 1.26 ± 0.8 (at B) and 0.83 ± 0.3 (at C) ($p < 0.01$). There was a significant correlation between changes in LVMI and myocardial collagen expression.**
*Conclusion:* **Complete reverse remodeling with regression of LVH and myocardial collagen protein expression can be anticipated after surgical correction of experimental aortic stenosis.**

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Aortic stenosis is the most common acquired heart valve disease in western civilization. It is associated with an increased left ventricular pressure load, leading to pathological ventricular remodeling and significant left ventricular hypertrophy (LVH). Although the reversal of LVH with normalization of left ventricular mass - termed reverse remodeling - can be anticipated after surgical therapy (1-3), the underlying cellular and molecular changes in the myocardium are not completely understood.

The development and eventual regression of LVH is a complex process leading to structural and molecular alterations of the myocardium, consisting of myocytes and the extracellular matrix (ECM). The ECM has a major role in preserving the mechanical properties and structural integrity of the myocardium. It consists of different collagens, mostly type I and III, as well as others including elastin, proteoglycans, fibroblasts, macrophages, and interstitial cells (4). As such, the heart has a three-dimensional extracellular fibrillar collagen scaffolding to sustain tissue integrity and an efficient pump function (5). Although the regression of myocardial interstitial fibrosis has been examined histologically after aortic valve surgery (1), gene expression of myocardial collagen has not been analyzed in the circumstance of myocardial reverse remodeling. As such, cardiac collagen type I and III are the two basic proteins that constitute the ECM and maintain myocardial integrity. Therefore, it is of major importance to analyze any potential reversal in collagen gene expression in order to understand the completeness of the myocardial reverse remodeling process after aortic valve surgery. The study aim was to analyze gene expression for myocardial collagen types I and III in LVH, as well as after ventricular reverse remodeling induced by definitive corrective surgical therapy for experimental aortic stenosis.

## Materials and methods

### Induction of LVH

LVH was induced using a standard experimental model of supracoronary banding in 44 growing female

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Merino sheep (age at baseline 6-8 months). Approval for animal use was obtained from the governmental offices, and all animals received human care in compliance with standard guidelines (6). General anesthesia was applied for all surgical interventions using scopolamine (0.03 mg/kg), xylazine (0.22 mg/kg) and ketamine (11 mg/kg) intramuscularly for induction, as well as endotracheal intubation, isoflurane (0.5-2 vol.%), fentanyl (0.1 mg) and pancuronium (1 mg).

During baseline operations (point A) the animals received supracoronary banding of the ascending aorta to induce LVH via a left lateral thoracotomy. After 8.3 ± 1.0 months, corrective surgical therapy (point B) mimicking aortic valve surgery in the clinical surroundings was performed. A median sternotomy was used, the band was resected, and normal ascending aortic blood flow restored. After another 10.1 ± 2.0 months, final examinations (point C) were performed to allow for complete regression of LVH and normalization of left ventricular mass.

**Echocardiography**

Several standard measurements including hemodynamic and echocardiographic examinations using transthoracic and transesophageal echocardiography were performed at points A, B, and C.

**Histology**

Sections of the left ventricular free wall were snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Thus, each animal served as its own individual control. Quantitative histological analyses were performed after hematoxylin and eosin staining, using an Axioplan2 (Carl Zeiss GmbH, Jena, Germany) microscope and KS 300 Imaging System 3.0 (Carl Zeiss Vision GmbH, Eching, Germany). Sirius red staining was performed on deparaffinized 5 µm-thick tissue sections. The tissue was exposed to pikro-Sirius red-solution (Hollborn & Söhne, Leipzig, Germany) for 1 h, washed, dehydrogenated, and finally embedded in Pertex (Histolab, Västra Frölunde, Sweden) mounting medium.

**Collagen gene expression**

Subtractive hybridization and alignment with a reference data bank (National Center for Biotechnology Information, USA) were used specifically to delineate the sequences for cardiac collagen types I and III. At invariant positions, sense and anti-sense primer (collagen I: sense GGTGCCCGTGGAAGTGAT, antisense CCAGGAAGCCCATTGGCT; collagen III: sense ATGGAGGGTGGTTTTCAGTTTAG, antisense AGGCTTTCTTCACATTCCCACT) were determined and applied. The amplified sequences were cloned using TOPO-TA cloning vector (Invitrogen, Groningen, Netherlands) and sequenced with an ABI 377 sequencer.

Frozen left ventricular tissue samples were prepared with an Ultra-Turrax™ tissue homogenizer using Trizol Reagent (Invitrogen, Karlsruhe, Germany). An aliquot of total RNA (500 ng) was reversely transcribed into cDNA using reverse transcriptase (Omniscript, Qiagen, Hilden, Germany) and random primers (Invitrogen). To quantify messenger RNA (mRNA) expression levels, an aliquot of the cDNA was used in the real-time PCR reaction containing the gene-specific primers and LightCycler-FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics, Mannheim, Germany). The specific expression levels were analyzed using LightCycler software and expressed in relation to the individual 18S rRNA expression (sense AGTAGGTCTGCCAGTAGCAT, antisense CTCATTCCAATTACAGGGCC) in relative units. Each PCR was performed three times, resulting in a variability of less than 10%.

Protein analyses were performed using a standard Western blot. For electrophoresis, 20 µg of total protein was separated in a sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) and blotted onto a nitrocellulose membrane (Roth, Karlsruhe, Germany). Membranes were blocked in 5% milk powder (Roth) in Tris-buffered saline with 0.5% Tween 20 for 1 h. After washing, membranes were incubated with goat anti human collagen I and III (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies for 2 h. A mouse anti human GAPDH (Hytest, Turku, Finland) was used as a reference. After washing, membranes were incubated with rabbit anti goat IgG secondary antibodies (regarding collagen I and III) or rabbit anti mouse IgG (regarding GAPDH), all conjugated with horseradish peroxidase, for 1 h (Sigma, Deisenhofen, Germany). Subsequently, membranes were developed with Super Signal Reagent (Pierce, Rockford, IL, USA).

**Statistical analysis**

Results were provided as mean ± SD. The SPSS software (SPSS Inc., Chicago, IL, USA) was used. All results were tested for normal distribution. For comparison of mean values at the different time points, a one-way ANOVA was performed. For post-hoc analysis the Bonferroni-test was applied. A p-value <0.05 on two-tailed testing was considered to be statistically significant.

## Results

Initial banding was performed successfully in all animals. During the time interval between banding (point A) and surgical correction (point B), eight of the 44 sheep (18.2%) died due to perforation of the ascending

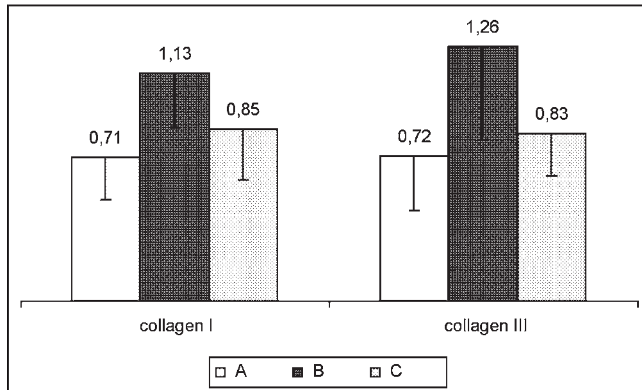


*Figure 1: Protein expression for cardiac collagen type I and III at baseline (A), after development of left ventricular hypertrophy (B), and after corrective surgical therapy (C). Results are given in relation to an internal standard in relative units. *p <0.01 versus previous time point.*

aorta (n = 3), heart failure (n = 3), a pericardial cyst (n = 1) or respiratory tract infection (n = 1). During surgical correction (point B), severe adhesions were found around the ascending aorta. Another nine sheep died perioperatively due to bleeding (n = 3), low cardiac output syndrome (n = 2), sternal wound infection (n = 3) or endocarditis (n = 1). The other 27 sheep survived until the end of the study and underwent final measurements (point C). Further results were provided for the 27 sheep that underwent measurements at points A, B, and C.

Morphological evaluations revealed an increase in body weight from 35.9 ± 5.5 (at A) to 54.1 ± 6.0 (at B) and 59.3 ± 7.0 kg (at C); during this time, all animals were fed a standard diet, ad libitum. The mean body surface area was 1.1 ± 0.1, 1.4 ± 0.1 and 1.5 ± 0.2 $m^2$ at points A, B, and C, respectively. Hemodynamic function as assessed by echocardiography revealed left ventricular ejection fractions of 66 ± 6, 69 ± 10 and 62 ± 8% at points A, B, and C, respectively (p = NS, while the respective cardiac indices were 3.5 ± 1.5, 4.2 ± 1.3,

*Table I: Maximum aortic pressure gradient ($P_{max}$), left ventricular mass index (LVMI) and myocardial fiber index at baseline (A), after development of left ventricular hypertrophy (LVH) (B), and after corrective surgical therapy (C).*

| Time point | $P_{max}$ (mmHg) | LVMI ($g/m^2$) | Myocardial fiber index ($\mu m/m^2$) |
|---|---|---|---|
| A (baseline) | 2.0 ± 0.2 | 82 ± 21 | 9.1 ± 1.2 |
| B (LVH) | 57 ± 12* | 150 ± 33* | 12.3 ± 1.4* |
| C (regression) | 3.0 ± 1.3* | 78 ± 18* | 8.4 ± 0.9* |

*p <0.01 versus previous time point.

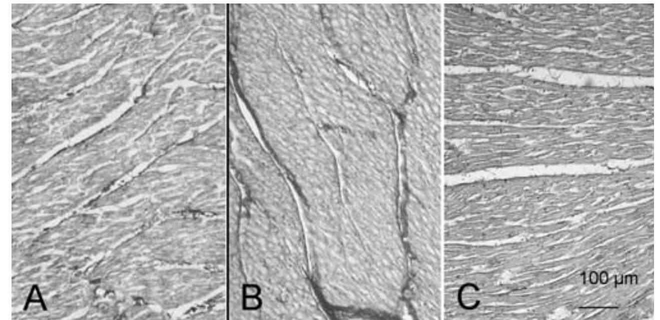


*Figure 2: Histology applying Sirius red collagen staining at baseline (A), after left ventricular hypertrophy (B), and after reverse remodeling (C). (Light microscopy, original magnification ×12.5.)*

and 4.2 ± 1.4 $l/min/m^2$ (p = NS). Throughout the study, circumferential wall stress was stable at 55 ± 19 (at A), 47 ± 35 (at B) and 57 ± 18 $dyne/cm^2$ (at C). Banding at point A resulted in an initial pressure gradient of 25.8 ± 6.3 mmHg that steadily increased parallel with growth, as diagnosed on serial follow up echocardiography. The myocardial fiber diameter was 11.3 ± 0.8, 15.9 ± 1.2, and 11.4 ± 1.0 µm at points A, B, and C (p <0.01). The LVMI, maximum pressure gradients and myocardial fiber diameter index are listed for baseline (A), after development of LVH (B) and after reversal of LVH (C) in Table I.

In order to monitor cardiac collagen I and III gene expression in an initial step, the unknown sequences were successfully identified for the sheep model. Collagen I mRNA levels were 1.41 ± 0.2 (at A) versus 0.5 ± 0.1 (at B) and 0.8 ± 0.1 (at C), while the respective collagen III mRNA levels were 0.8 ± 0.2, 0.7 ± 0.1, and 1.0 ± 0.1. Protein expression for collagens I and III for the three different time points is illustrated in Figure 1. In comparison to baseline, there was a significant increase in collagen I and III protein expression after development of LVH (at B), but reversal of all these changes was observed after surgical treatment (at C). Collagen staining revealed a clear increase in parallel to LVH (at B) and a decrease after reverse remodeling (at C) (Fig. 2).

The association between LVMI and cardiac collagen type I and III protein expression is illustrated graphically in Figure 3. There was a parallel increase of LVMI as well as cardiac collagen type I and III protein expression between the time points A and B. After surgical therapy, between points B and C there was a parallel decrease in these parameters. For the animal model there was a good correlation between changes in LVMI and cardiac collagen protein expression (see Fig. 3).

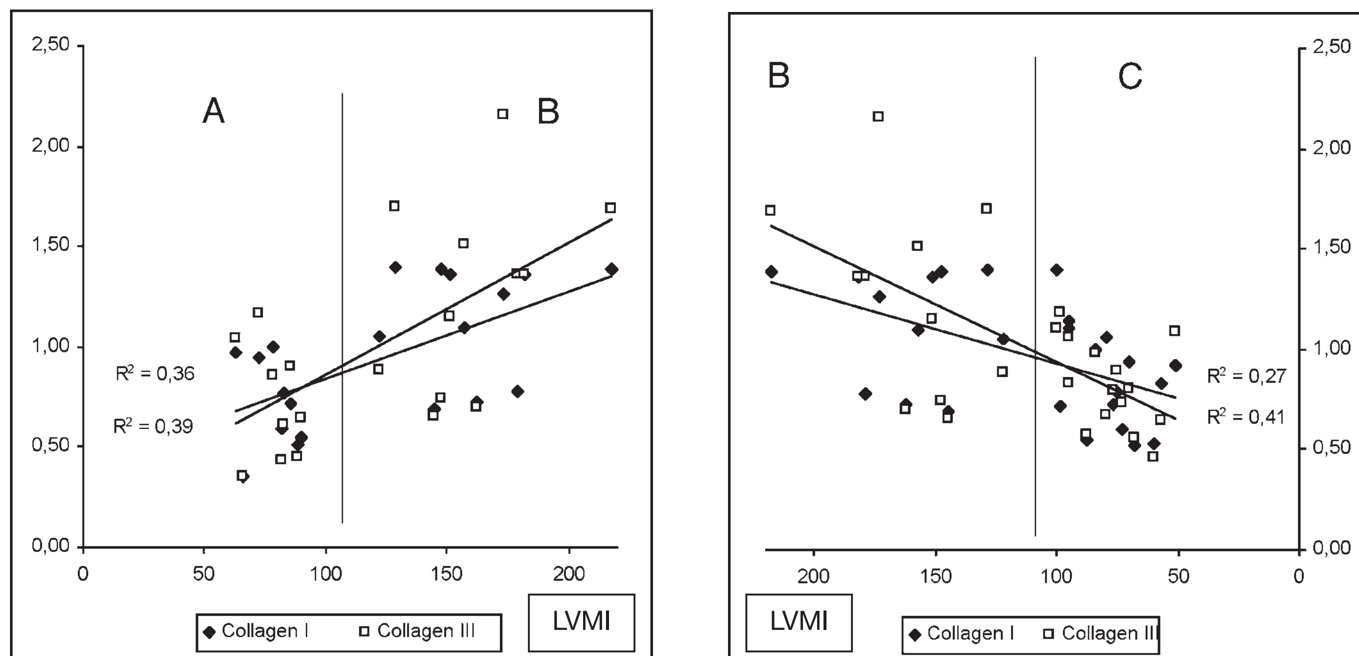


*Figure 3: Association of cardiac collagen gene expression (ordinate in relative units) and left ventricular mass index (LVMI; g/m²) at baseline (A), after development of left ventricular hypertrophy (B), and after corrective surgical therapy (C).*

## Discussion

Aortic valve surgery is a routine procedure leading to an instantaneous restoration of close to physiological transvalvular hemodynamics. During the further postoperative course, relief of the pressure load results in left ventricular reverse remodeling. Reverse remodeling, with normalization of the left ventricular myocardial ultrastructure, is especially important because myocardial remodeling is a major determinant for patient outcome, being associated with increased morbidity and mortality (7-9). A major aim of the present study was to determine whether complete reverse remodeling could occur on the molecular level after corrective surgical therapy for aortic stenosis. As such, the evaluation of cardiac collagen protein expression is of special interest as collagen is the most frequent protein end-product composing the cardiac ECM. The collagen scaffold guarantees the integrity and function of the entire myocardium (5).

Several important findings arose in the present study. In the controlled model, myocardial gene expression was inducible, and this led to significant increases in cardiac collagen type I and III protein expression in parallel with induction of compensated LVH. After corrective surgical therapy, complete reverse remodeling associated with complete regression of cardiac collagen type I and type III protein expression was found. Furthermore, in the animal model a direct correlation between changes in LVMI and myocardial collagen protein expression was documented after corrective surgical therapy.

Several aspects regarding the experimental model should be discussed. The atypical coronary perfusion pattern associated with supracoronary banding was taken into account in order to obtain a rather simple model ensuring survival of the animals. Clearly, this does not completely model the clinical situation of aortic stenosis, and possible influences of the differing effects on coronary flow imposed by supracoronary banding cannot be excluded. The use of growing sheep during the study was important in order to achieve a gradual increase in pressure gradient after banding. From the hemodynamic findings it became clear that all animals were in a compensated hemodynamic situation, mimicking the clinical situation of compensated aortic stenosis. Thus, no conclusions can be drawn on patients with myocardial decompensation leading to heart failure. One limitation of the sheep model was that the delineation of specific gene sequences was very time-consuming, and very few data on suitable antibodies for molecular analyses were available. However, protein expression for the cardiac collagens targeted were eventually determined. The results of mRNA analyses were not consistent with the final findings on protein expression. However, there may be regulatory mechanisms leading to a decreased mRNA expression due to previously increased collagen protein generation at the specific time point examined. Histological analyses (see Fig. 2) clearly underlined a significant change in collagen content in parallel to LVH (point B) and a regression in parallel to ventricu-

lar reverse remodeling (point C).

The question remains, however, as to the nature of the potential mechanisms leading to reverse remodeling of the ventricle, and the factors involved after aortic valve surgery. Myocardial remodeling, as well as reverse remodeling, after therapeutic intervention for aortic stenosis are complex processes that have multifactorial causes. This includes changes in pressure load conditions, an interplay of different regulatory systems of the body, and the specific function of certain growth effectors and inhibitors. Relief of the pressure gradient most certainly is the initial trigger for reverse remodeling to occur. The most obvious intrinsic regulatory mechanism affecting cardiac collagen content is the renin-angiotensin system (RAS) (10,11). Furthermore, the matrix metalloproteinases (MMPs) and their tissue inhibitors are also involved, as they have specific effects on ECM composition and turnover (12). Growth factors such as the insulin-like growth factor system are also involved. The impact of the RAS on myocardial remodeling has been outlined in several studies (13-21), and angiotensin has been shown to be involved specifically in fibrinogen formation. Previously, a close correlation has also been demonstrated between RAS gene expression and myocardial hypertrophy, even during reverse remodeling (22). With regard to MMPs and their tissue inhibitors, an increased gene expression in parallel to experimental LVH as well as regression to baseline after corrective surgical therapy has also been documented previously (23). Thus, both the RAS and the MMPs may have a major impact on the regulation of myocardial collagen content, on LVH, and on left ventricular reverse remodeling. However, a cause and effect relationship could not be proven in the present study.

In future, additional medical therapies (e.g., the use of ACE inhibitors or aldactone) to additionally affect the cardiac ECM may evolve as a useful therapeutic strategy to support reverse left ventricular remodeling after aortic valve surgery. As shown previously (1), reverse remodeling of the left ventricle in not even complete after 10 years. In theory, it is important to operate on patients with valvular heart diseases relatively early in order to prevent decompensation of the myocardium due to pressure or volume overload. It is also important to achieve complete reverse remodeling of the left ventricle, usually within the first year after corrective surgery, with the process probably being supported by ACE inhibitors and aldactone. Surgical restoration of hemodynamic function with relief of the pressure gradient will remain the most important therapeutic strategy.

*In conclusion*, by using a model of compensated LVH, it was shown that myocardial remodeling as well as cardiac collagen type I and III protein expression are reversible after corrective surgical therapy. Complete restitution of myocardial protein expression is possible after surgical therapy, and this should result in complete normalization of myocardial ultrastructure. Thus, aortic valve surgery may be considered a curative therapy, leading to restitutio ad integrum.

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