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In Vivo Aortic Valve Thermal Heterogeneity in Patients With Nonrheumatic Aortic Valve Stenosis

The First In Vivo Experience in Humans

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Objectives We investigated in vivo in aortic valve stenosis (AVS) whether there is: 1) thermal heterogeneity within the valve leaflets; 2) temperature difference between the leaflets and the ascending aortic wall; and 3) a possible correlation between heat production, inflammation, and neoangiogenesis.

Background Histological studies have demonstrated a potential role of inflammation and neoangiogenesis in AVS.

Methods We examined 96 leaflets scheduled for aortic valve replacement. Twenty-five patients had AVS, and 7 had aortic valve insufficiency (AVI). Temperature measurements were performed right before hypothermic cardioplegia. Temperature difference (ΔT) was assigned as the mean temperature of each leaflet minus the temperature of the aortic wall. Histological, immunohistological analysis, and vascular endothelial growth factor (VEGF) immunoreactivity was performed.

Results Significant thermal heterogeneity was recorded within the leaflets of AVS, compared with AVI ($1.52 \pm 1.35°C$ vs. $0.13 \pm 0.11°C$, $p < 0.01$). In AVS ΔT was greater in all leaflets compared with the AVI group ($p < 0.01$). Leaflets of AVS had increased inflammatory cell infiltration, calcium deposit, and anti-VEGF expression compared with AVI ($p < 0.01$).

Conclusions Thermal heterogeneity is increased in AVS and correlates with inflammatory mononuclear cell infiltration, expression of pro-inflammatory cytokines and neoangiogenic factors. (J Am Coll Cardiol 2008;52:758–63)

The prevalence of aortic valve stenosis (AVS) increases with age and is the most common reason for valve replacement (1–4). Several studies have shown that local infiltration of inflammatory cells within leaflets of AVS is a prominent feature of aortic valve calcification. In addition to inflammation, neoangiogenesis has been described in severe AVS, similarly to atherosclerotic lesions (3,5).

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We postulated that the inflammatory activation within the aortic valves might generate heat (6,7). We investigated in vivo in patients with AVS whether there is: 1) thermal heterogeneity within the leaflets; 2) temperature difference between the leaflets and the ascending aortic wall; and 3) a possible correlation between heat production, inflammation, and neoangiogenesis.

Methods

Study population. We examined 96 leaflets scheduled for aortic valve replacement. Twenty-five patients had AVS, and 7 had aortic valve insufficiency (AVI) (control group). Patients with known coronary artery disease, ejection fraction <50%, rheumatic fever in their medical history, intercurrent inflammatory or neoplastic condition, or those medicated with anti-inflammatory drugs except for aspirin were excluded from the study. Cardiac catheterization was performed to exclude the presence of coronary artery disease (stenoses ≥50%). The Ethics Committee of all institutions approved the study protocol.

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Procedure. After cardiopulmonary bypass was started, the ascending aorta was cross-clamped. Temperature measurements were obtained at this stage (see temperature measurements in the following text) right before the hypothermical cardioplegia.

In vivo measurements. A thermographic catheter with a sensitive thermistor at the distal tip was used (BetaTHERM sensor, Galway, Ireland). After preparation of the field for clear visualization of the valve leaflets and exclusion of macroscopically atherosclerotic lesions on the aortic wall, the thermistor was placed on each leaflet 5 times, and the maximal, minimal, and mean temperature was obtained. The thermistor was applied on the internal aortic wall 5 times, and the average temperature was assigned as the aortic wall temperature. "Thermal heterogeneity" within the leaflets was assigned as the maximal temperature between the leaflets minus the minimal temperature between the leaflets.

"Temperature difference" (ΔT) was assigned as the mean temperature of each leaflet minus the mean temperature of the aortic wall. "Maximal ΔT" was assigned as the maximal temperature between the leaflets minus the temperature of the aortic wall. Aortic valves were then harvested.

Ex vivo measurements. In 10 additional valves of patients with AVS, ex vivo temperature measurements were performed, immediately after extraction, in a humidified incubator (SANYO, Osaka, Japan) at 37°C in normoxic conditions.

Histological and immunohistochemical analysis. All samples were fixed in 10% buffered formalin for 24 h, decalcified overnight with formic acid, and processed for routine paraffin embedding. Each leaflet was then divided radially, and 5-μm-thick sections were obtained from paraffin-embedded samples and stained with hematoxylin-eosin staining (3). Histological sections were analyzed semiquantitatively as previously described (5,8,9).

Table 1 Baseline Demographic and Clinical Characteristics

	AVS	AVI	p Value
Clinical variables			
Age, yrs (mean ± SD)	68.2 ± 10.52	59.14 ± 13.52	0.06
Male gender	18 (72%)	5 (71.4%)	0.99
Hypertension	16 (64.0%)	4 (57.1%)	0.99
Hyperlipidemia	8 (32.0%)	3 (42.8%)	0.67
Diabetes mellitus	6 (24%)	2 (28.5%)	0.99
Smoking status	8 (32.0%)	3 (42.8%)	0.67
EF (%)	59.04 ± 6.68	59.57 ± 4.61	0.84
Previous medication			
ACE-Is	17 (68.0%)	4 (57.1%)	0.67
Statins	8 (32.0%)	3 (42.0%)	0.67
Calcium-channel blockers	6 (24.0%)	3 (42.0%)	0.37
Beta-blockers	6 (24.0%)	2 (28.5%)	0.99
Diuretics	6 (24.0%)	1 (14.3%)	0.99

ACE-I = angiotensin-converting enzyme inhibitor; AVI = aortic valve insufficiency; AVS = aortic valve stenosis; EF = ejection fraction.

Abbreviations and Acronyms

AVI = aortic valve insufficiency
AVS = aortic valve stenosis
CD3 = cluster of differentiation 3
IL = interleukin
TNF = tumor necrosis factor
VEGF = vascular endothelial growth factor
ΔT = temperature difference

Immunohistochemical staining for cluster of differentiation 3 (CD3), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and vascular endothelial growth factor (VEGF) was performed by standard immunohistochemistry including antigen retrieval. The following primary antibodies were used: anti CD3, (Dako, Glostrup, Denmark), anti-TNF-α, (RnD, Minneapolis, Minnesota), anti–IL-6, (RnD), anti-VEGF, (Becton Dickinson, Franklin Lakes, New Jersey). For negative controls, the primary antibody was omitted and replaced by nonimmune serum. Finally, slides were counterstained with hematoxylin and mounted.

Evaluation of hematoxylin-eosin staining and immunohistochemistry was performed independently and blindly on the groups included by 2 pathologists. For the evaluation of immunohistochemistry, CD3 staining was graded according to the degree of inflammation. The expression of TNF-α and IL-6 was graded according to previous studies (10). The VEGF quantification was performed semiquantitatively as previously described (11).

Statistical analysis. Statistical analysis was performed with the commercially available software (SPSS Inc., Chicago, Illinois). Quantitative data are presented as rates or mean value ± SD. Probability values are 2-sided from the Student *t* test for continuous variables. Noncontinuous values were compared by the chi-square test. For all histological and immunohistological measurements, interobserver and intraobserver variability was assessed by reanalysis of a representative sample of histological sections. A value of $p < 0.05$ was considered significant.

Results

Baseline demographic and clinical characteristics. The procedure was safe and without complications. The baseline clinical and demographic characteristics are demonstrated in Table 1.

Thermographic findings. IN VIVO MEASUREMENTS. Significant thermal heterogeneity was recorded in the AVS compared with the AVI group. Mean and maximal ΔT was greater in the AVS compared with the AVI group (Table 2).

Table 2 In Vivo ΔT of Aortic Valves in Patients With AVS and AVI

	Thermal Heterogeneity	Mean ΔT	Maximal ΔT
AVS (°C)	1.52 ± 1.35	1.28 ± 0.93	1.96 ± 1.16
AVI (°C)	0.13 ± 0.11	0.06 ± 0.15	0.13 ± 0.13
p value	0.01	<0.01	<0.01

ΔT = temperature difference; other abbreviations as in Table 1.

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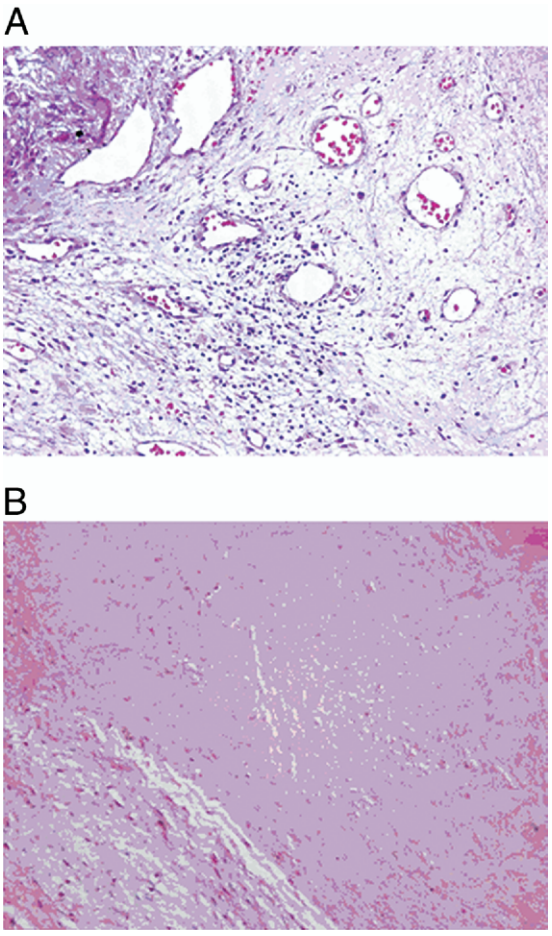

Figure 1 Representative Photomicrographs of HE Staining in Examined Specimens of Aortic Valves

(A) Hematoxylin and eosin (HE) staining in aortic valve stenosis showing intense inflammation (×200). **(B)** The HE staining in aortic valve insufficiency showing normal valvular stroma with sparse cellularity (×200).

In AVS leaflets, ΔT was greater compared with the AVI group (noncoronary: 1.09 ± 1.16°C vs. 0.10 ± 0.10°C; right: 1.24 ± 1.03°C vs. 0.02 ± 0.19°C; left: 1.53 ± 1.23°C vs. 0.05 ± 0.19°C; $p < 0.01$ for all comparisons). There was no difference in ΔT between the 3 leaflets in both groups (AVS: $p = 0.67$, AVI: $p = 0.72$). When analyzed by statin intake, mean ΔT did not differ between the 2 groups ($p = 0.49$).

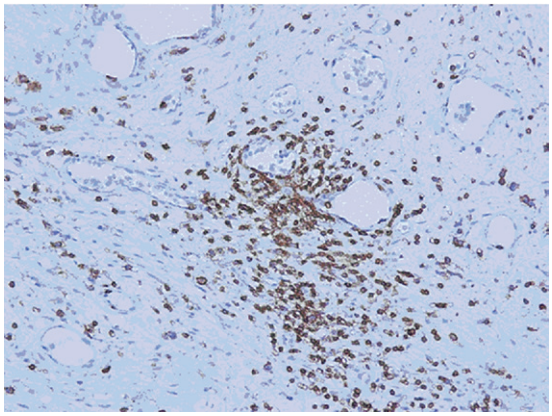

Figure 2 Representative Photomicrograph of CD3-Positive Leukocytes in Examined Specimens of Aortic Valves

Intense expression of cluster of differentiation 3 (CD3)–positive leukocytes in aortic valve stenosis.

EX VIVO MEASUREMENTS. Thermal heterogeneity before and after excision was not significant (1.15 ± 0.75°C vs. 1.07 ± 0.73°C, $p = 0.40$).

Histological analysis. There was no significant difference in the interobserver and intraobserver readings. Inflammatory infiltrates were present in all samples, in small aggregate areas around substantial calcium deposits or diffused throughout the leaflets, on the aortic or ventricular side of the subendothelial layers. The T-lymphocytes, monocytes, and plasma cells were the most predominant cells in these infiltrates. Dense lymphocyte infiltration was observed in 97.8% of AVS versus 0% of AVI ($p < 0.01$) (Fig. 1). Increased infiltration from mast cells and calcium deposit was detected in all leaflets from AVS versus none from AVI ($p < 0.01$) (Table 3).

Immunohistochemistry. All specimens from AVS were positive for CD3-, TNF-α, and IL-6, in contrast to

Table 3 Immunohistological Analysis of Aortic Valve Leaflets Between the 2 Groups of Patients

	AVS (n = 75)				AVI (n = 21)			
Grade	0	1+	2+	3+	0	1+	2+	3+
Histological features, n (%)								
T lymphocytes	0 (0%)	2 (2.6%)	9 (12%)	64 (85.4%)	19 (90.4%)*	2 (9.6%)	0 (0%)*	0 (0%)*
Mast cells	0 (0%)	0 (0%)	10 (14%)	65 (86%)	17 (81%)*	4 (9%)*	0 (0%)*	0 (0%)*
Calcium deposits	0 (0%)	0 (0%)	2 (3%)	73 (97%)	16 (76%)*	5 (23%)*	0 (0%)	0 (0%)*
Immunohistological features, n (%)								
CD3	0 (0%)	1 (1.3%)	8 (10.6%)	66 (88%)	17 (80.9%)*	4 (19.0%)*	0 (0%)	0 (0%)*
TNF-α	0 (0%)	2 (2.7%)	12 (16%)	61 (81.3%)	16 (76.2%)*	5 (23.8%)*	0 (0%)*	0 (0%)*
IL-6	0 (0%)	2 (0%)	13 (17.3%)	60 (80%)	17 (80.9%)*	4 (19.0%)*	0 (0%)*	0 (0%)*

*$p < 0.05$ for comparisons between aortic valve stenosis (AVS) and aortic valve insufficiency (AVI).
CD3 = cluster of differentiation 3; IL = interleukin; TNF = tumor necrosis factor.

negative specimens of the AVI group (Figs. 2 to 4). Intense expression of inflammatory indexes was found in the AVS compared with the AVI group (Table 3). We categorized specimens into 2 groups with a cut-off value of 2 on the basis of the semiquantitative scale for CD3, TNF-α, IL-6, calcium deposit, mast cell, and T lymphocyte expression. Mean ΔT was greater in leaflets with intense compared with low expression of CD3, TNF-α, and IL-6 (CD-3: 1.26 $\pm$ 1.11°C vs. 0.51 $\pm$ 1.01°C; TNF-α: 1.38 $\pm$ 1.13°C vs. 0.39 $\pm$ 0.83°C; IL-6: 1.35 $\pm$ 1.12°C vs. 0.49 $\pm$ 0.95°C, $p < 0.01$ for all comparisons) (Figs. 5A to 5C).

Mean ΔT was greater in leaflets with intense compared with low expression of calcium deposit (1.33 $\pm$ 1.11°C vs. 0.02 $\pm$ 0.25°C, $p < 0.01$). In leaflets with AVS and intense expression of mast cells, mean ΔT was greater compared with leaflets with low expression (1.52 $\pm$ 1.01°C vs. 0.26 $\pm$ 0.67°C, $p < 0.01$). In leaflets with AVS and intense expression of T lymphocytes, mean ΔT was greater compared with leaflets with low expression (0.54 $\pm$ 1.00°C vs. 0.21 $\pm$ 0.66°C, $p < 0.01$).

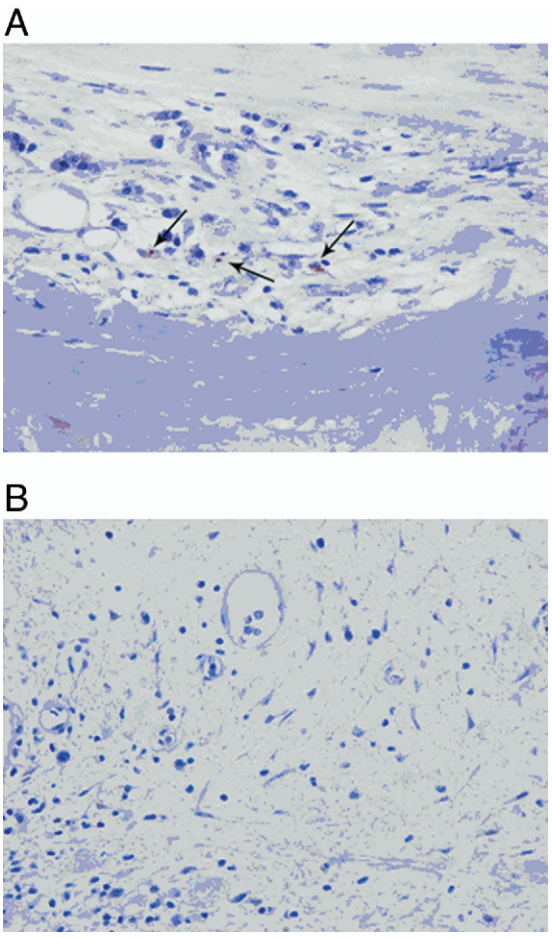

Figure 3 Representative Photomicrographs of Anti–TNF-α Staining in Examined Specimens of Aortic Valves

(A) Anti–tumor necrosis factor (TNF)-α staining in aortic valve stenosis. **Arrows** indicate positivity to anti–TNF-α. **(B)** Anti–TNF-α staining in aortic valve insufficiency showing negative staining for anti–TNF-α (×400).

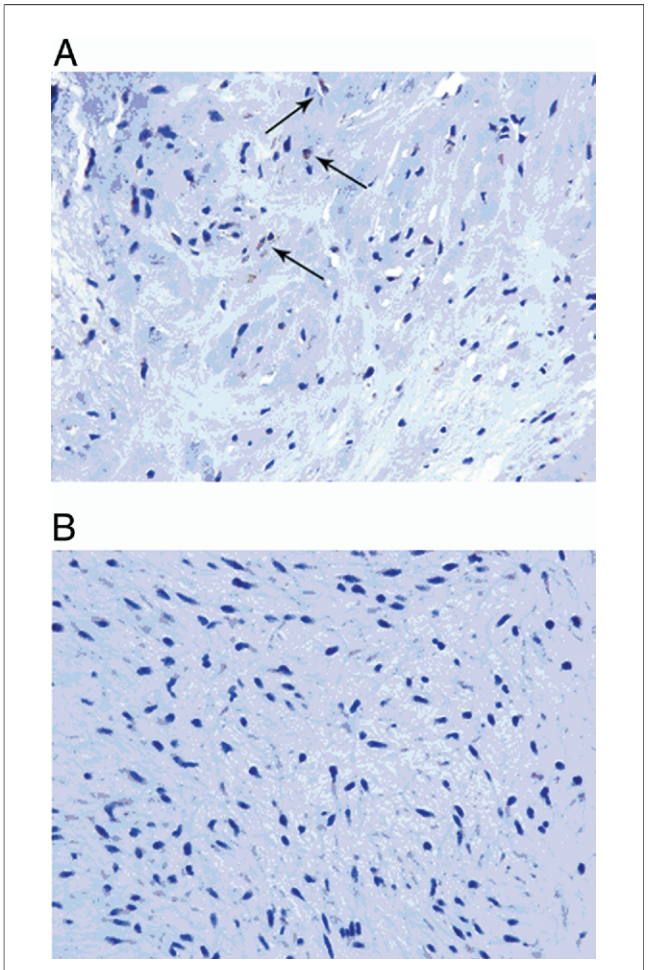

Figure 4 Representative Photomicrographs of IL-6 Staining in Examined Specimens of Aortic Valves

(A) Interleukin (IL)-6 staining in aortic valve stenosis. **Arrows** indicate IL-6 immunopositive inflammatory cells in the valve stroma (×400). **(B)**. The IL-6 staining in aortic insufficiency showing negative staining for IL-6 (×400).

VEGF immunoreactivity. Neoangiogenesis was evident in all AVS specimens, in areas where inflammatory infiltrates were denser, in contrast to AVI (Fig. 6, Table 4). Mean ΔT was greater in leaflets with a high compared with a low degree of VEGF immunoreactivity (1.28 $\pm$ 1.14°C vs. 0.18 $\pm$ 0.38°C, $p < 0.01$) (Fig. 5D).

Discussion

The main finding of this study is that nonrheumatic aortic stenotic valves have in vivo significant thermal heterogeneity that correlates with inflammatory infiltrates and neovessels, supporting the hypothesis that an active inflammatory process is involved.

Calcific aortic valve disease has been recognized as an active process with features of chronic inflammation (12). We therefore evaluated local inflammatory process at the

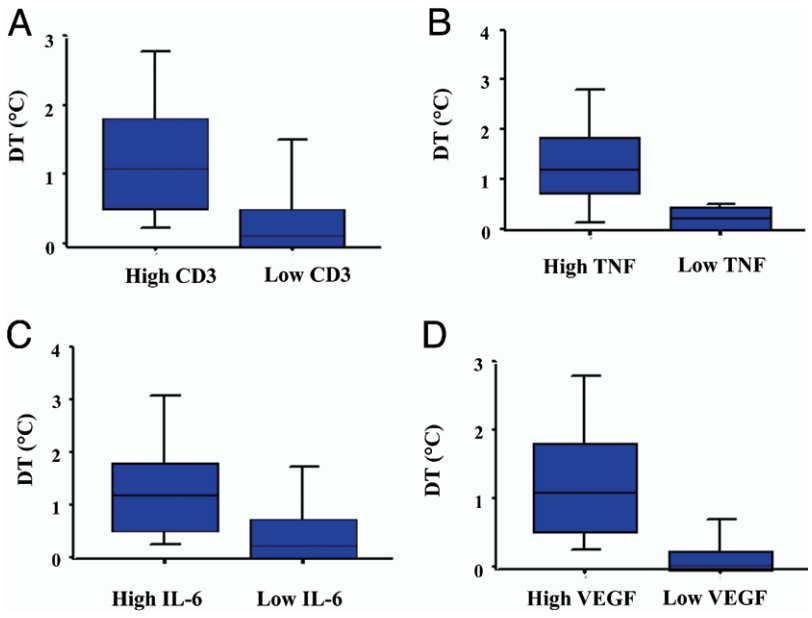

Figure 5 DT in Leaflets With Intense Versus Low Expression of Inflammatory Indexes

Mean temperature difference (DT) in leaflets with intense versus leaflets with low expression of CD3 **(A)**, TNF-α **(B)**, IL-6 **(C)**, and vascular endothelial growth factor (VEGF) **(D)**. The **bottom of the boxes** represents the first quartile; the **top of the boxes** represents the third quartile, and the **line in the box** represents the median value. Abbreviations as in Figures 2, 3, and 4.

leaflets of AVS by thermography, because it is the only method currently available to measure heat generation reflecting inflammation (13,14). According to our results there is a significant difference in local inflammatory activation within aortic valve leaflets as well as between the leaflets of the same stenotic valve. This difference was associated with increased inflammatory cell infiltration, such as T-lymphocytes and mast cells, in accordance with previous observations (15). Other studies have shown that increased inflammatory markers are attributed to shared risk factors with cardiovascular disease and infiltration of lipoproteins and interstitial cells (16,17). Inhibition of the inflammatory activation, possibly by statins (18,19) although not demonstrated in the current study, might be a beneficial pharmacological prevention of AVS.

Study limitations. We performed ex vivo measurements from AVS specimens to verify that temperature measurements were not altered right before hypothermical cardioplegia. In vivo measurements during cardiac catheterization would be the ideal method; however, the impact of the cooling effect of blood flow on thermography could not have been eliminated (20). Furthermore, we used as a reference temperature the temperature of the aortic wall. Although we macroscopically selected areas without atherosclerotic lesions, we cannot exclude the presence of small lesions with inflammatory activation, leading to underestimation of the temperature difference between the aortic wall and aortic valve. In addition, further subgroup analysis is limited by the small number of stenotic aortic valves with low inflammatory activation. Finally, patients with AVI comprised the control group, because "normal" heart valves cannot be used.

Clinical implications. The present study supports the hypothesis that an active inflammatory process is involved in AVS. Anti-inflammatory treatment and statin intake in such patients might have significant prognostic and therapeutic implications. Future studies need to be performed for further evaluation.

Conclusions

Nonrheumatic aortic stenotic valves have in vivo significant thermal heterogeneity reflecting an active local inflammatory activation. Neoangiogenesis, local inflammatory activation, and thermal heterogeneity are increased in AVS. Further studies need to be performed to investigate whether prevention of neoangiogenesis might have significant prognostic and therapeutic implications.

Table 4 Semiquantitative VEGF Immunoreactivity Between Leaflets of Patients With AVS and Patients With AVI

	AVS (n = 75)	AVI (n = 21)
	0 (0%)	16 (76.2%)*
++	0 (0%)	5 (23.8%)*
+++	4 (5.3%)	0 (0%)*
++++	6 (8.0%)	0 (0%)*
+++++	65 (86.6%)	0 (0%)*

*$p < 0.05$ for comparisons between aortic valve stenosis (AVS) and aortic valve insufficiency (AVI).
VEGF = vascular endothelial growth factor.

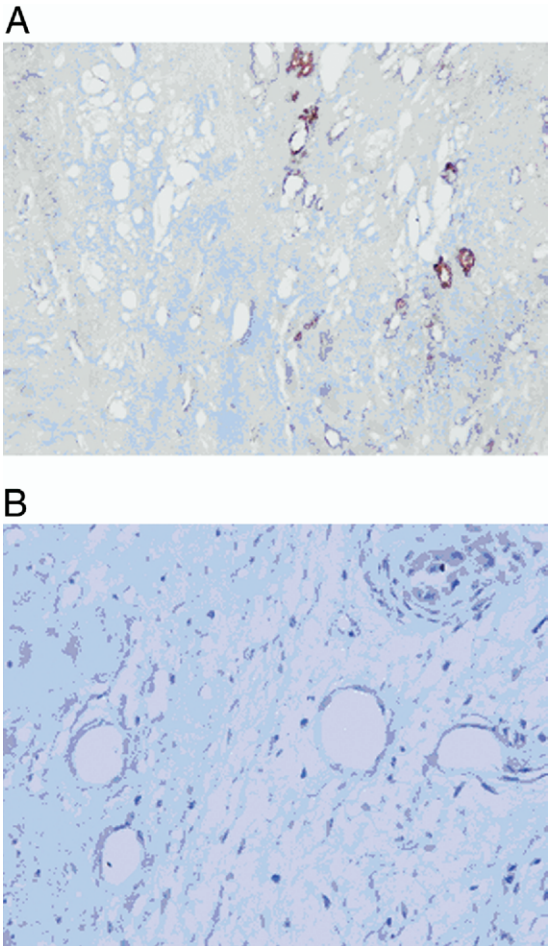

Figure 6 **Representative Photomicrographs of VEGF Staining in Examined Specimens of Aortic Valves**

(A) Marked vascular endothelial growth factor (VEGF) immunoreactivity in neovessels in severe aortic stenosis (×400). **(B)** Aortic valve insufficiency showing no VEGF immunoreactivity (×400).

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Key Words: aortic valve ■ inflammation thermography.