Vascular abnormalities and cardiomyocyte lipofuscin deposits in endomyocardial biopsy specimens of heart transplant recipients: Are they related to the development of cardiac allograft vasculopathy?

Michael Zakliczynski, MD,^a Jerzy Nozynski, MD,^b Dominika Konecka-Mrowka, PhD,^b
Anna Krynicka-Mazurek, MD,^c Marcin Swierad, MD,^d Marcin Maruszewski, MD,^a
Roman Przybylski, MD,^a and Marian Zembala, MD, PhD^a

Objective: The aim of this study was to assess the predictive value of microvascular abnormalities and lipofuscin observed in endomyocardial biopsy samples for the development of cardiac allograft vasculopathy.

Methods: The study group consisted of 68 cardiac allograft recipients (63 men and 5 women, 43 ± 12 years old). We performed a re-evaluation of 1071 endomyocardial biopsy specimens to search for microvascular diseases and lipofuscin in cardiocytes. Endomyocardial biopsy specimens with an International Society for Heart and Lung Transplantation rejection grade of 2 or more and those without arterioles were excluded. Abnormalities found in the remaining 517 specimens were correlated with the grade of rejection. Biopsy specimens obtained 2 weeks, 12 months, and 36 months after transplantation were compared with coronary angiography results, clinical events of cardiac allograft vasculopathies, and survivals. Kaplan–Meier curves were constructed to compare the time to the development of cardiac allograft vasculopathy or death.

Results: Enlarged endothelial cells, lymphocytes inside the arteriolar wall, occluded arteriolar lumen, endothelial vacuolization, and hypertrophy of the vascular muscle were significantly correlated with rejection grade. Although none of the vascular abnormalities predicted cardiac allograft vasculopathy, patients with lipofuscin deposits at the 12-month biopsy specimens were characterized by the rapid development of angiography-confirmed cardiac allograft vasculopathy ($P < .08$) and events related to cardiac allograft vasculopathy ($P < .03$, log–rank test).

Conclusion: Microvascular abnormalities correlate with mild cellular rejection, but they do not seem to be predictive for development of cardiac allograft vasculopathy detected by angiography. The presence of lipofuscin in 12-month endomyocardial biopsy specimens may be predictive of development of angiographically confirmed cardiac allograft vasculopathy.

Supplemental material is available online.

Cardiac allograft vasculopathy (CAV) is, apart from malignancy, the predominant cause of death in heart transplant recipients 3 or more years after heart transplantation.¹ Intimal hyperplasia, which begins in the narrowest parts of distal arterioles, and shrinkage of the whole vessel are the most char-

acteristic features of coronary artery lumen loss. However, a typical atherosclerotic plaque localized in the large epicardial vessels is also a frequent type of lesion.^{2,3} Despite significant differences between CAV features in large and small coronary vessels, an attempt is being made to find a common pathogenic mechanism for transplanted heart coronary artery disease, in which endothelial damage and inflammatory processes stimulating myofibroblast proliferation and fibrosis are the pivotal processes.⁴ The influence of nonimmunologic risk factors on the development of disease in transplanted heart coronary arteries is well documented,^{5,6} whereas evidence connecting acute rejection and CAV is rather weak.^{1,7-9} The main problem is an inability to separate the immunologic phenomena characterizing rejection from the pro-atherosclerotic side effects of high-dose immunosuppressive therapy, which has to be increased to control rejection.

There is no ideal method for monitoring CAV. Although coronary angiography (CAG) is not sensitive enough for diagnosing intimal hyperplasia, it is the most frequently used clinical tool for diagnosing CAV. Intravascular ultrasound is able to show the vessel wall structure, but it is limited to relatively large coronary arteries owing to the size of the

From the Department of Cardiac Surgery & Transplantation,^a Department of Pathology,^b and First Department of Cardiology,^d Silesian Center for Heart Disease, Zabrze, Poland; and the First Department of Internal Medicine,^c Third District Hospital of Upper Silesia, Rybnik, Poland.

This study was sponsored by the Polish Ministry of Science and Higher Education Grant No. 2P05C 059 30.

Received for publication Nov 22, 2008; revisions received Feb 10, 2009; accepted for publication Feb 22, 2009.

Address for reprints: Michael Zakliczynski, MD, Department of Cardiac Surgery & Transplantation, Silesian Center for Heart Disease, ul Szpitalna 2, 41-800 Zabrze, Poland (E-mail: m.zakliczynski@scs.pl).

J Thorac Cardiovasc Surg 2009;138:215-21
0022-5223/\$36.00

Copyright © 2009 by The American Association for Thoracic Surgery
doi:10.1016/j.jtcvs.2009.02.040

Abbreviations and Acronyms

AR	= acute cellular rejection
CAG	= coronary angiography
CAV	= cardiac allograft vasculopathy
EMB	= endomyocardial biopsy
ISHLT	= International Society for Heart and Lung Transplantation
OHT	= orthotopic heart transplantation

ultrasound probe. Owing to the high cost, the technical complexity, and the need for higher doses of nephrotoxic contrast substances, intravascular ultrasound remains a research tool rather than a clinical practice. In routine endomyocardial biopsy (EMB), a variety of pathomorphologic changes in small cardiac vessels can be observed. The presence of arterioles in EMB samples is incidental; however, an analysis of microvascular alterations may shed light on CAV pathogenesis. Some articles suggest a leading role for immunologic factors in the development of small vessel disease of the transplanted heart.^{10,11}

There is a growing interest in the role of aging markers in the pathogenesis of heart failure, including disease of the transplanted heart. Lipofuscin, an age pigment, is a nondegradable intralysosomal polymeric substance composed of oxidized proteins, lipids, and trace amounts of metals. It is mostly accumulated in long-living cells, such as cardiomyocytes and neurons, as a result of normal cell metabolism. Cell turnover is increased by stress factors, such as ischemia or inflammation, which consequently accelerate the accumulation of lipofuscin and other waste material, but no immunologic mechanism for the increase in lipofuscin deposit formation has yet been described.^{12,13} Autophagy, an intralysosomal degradation of a cell's own organelles, plays an important role in lipofuscin accumulation and cell renewal.¹⁴

The aim of this study was to determine whether vascular abnormalities and lipofuscin deposition in the cytoplasm of cardiomyocytes have any predictive value for the development of CAV.

MATERIAL AND METHODS

We re-evaluated all available EMB slides of patients in whom heart transplantation was performed in the Silesian Center for Heart Disease before the end of 1995, specifically searching for abnormalities in small arterial coronary vessels and lipofuscin deposits in the cytoplasm of cardiomyocytes.

Inclusion criteria were as follows: orthotopic heart transplantation (OHT) performed before the end of 1995, EMB performed 2 weeks after OHT and at the 12th and 36th months after OHT in patients eligible from the study, and at least one CAG performed after OHT. Among 197 patients who underwent OHT from 1986 to 1995, 68 patients were enrolled for this retrospective study. The majority of the remaining patients were excluded owing to early loss and the lack of at least one CAG. The study group consisted of 63 men and 5 women, with a mean age of 43 ± 12 years. All donors were below 35 years old, and head trauma was a predominant cause of death. The presence of the following "classic" risk factors for atheroscle-

rosis was identified in the study group: male sex in 63 (93%) patients, age over 40 years in 47 (69%) patients and age over 50 years in 21 (31%) patients, ischemic cardiomyopathy as an indication for OHT in 35 (51%) patients, body mass index over 25 kg/m^2 in 34 (50%) patients, diabetes mellitus before and/or after OHT in 11 (16%) patients, arterial hypertension in 30 (44%) patients, and some form of hyperlipidemia in 45 (66%) patients.

Immunosuppression was based on a triple-drug protocol with cyclosporine A, azathioprine, and prednisone. No elective cytolytic induction therapy was used. Mycophenolate mofetil, available since 1996, replaced azathioprine after the second episode of significant acute cellular rejection (AR). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) were used only on diagnosis of hyperlipidemia.

Elective EMB specimens were obtained at 1, 2, 3, 4, 6, and 8 weeks and 3, 6, 9, 12, and 18 months after OHT. The overall number of specimens considered for the purpose of this study was 1071.

AR was assessed by the grading system developed by the International Society for Heart and Lung Transplantation (ISHLT), with grades of 3A or greater recognized as significant and treated with bolus doses of methylprednisolone succinate (SoluMedrol), 1.0 g daily for 3 days, and subsequent therapy with a tapered dose of prednisone, starting at 1.0 mg/kg of body weight. The following numeric values for ISHLT grades were used in the analyses: 0 = 0 point; 1A = 1 point; 1B = 2 points; 2 = 3 points; 3A = 4 points; 3B = 5 points; and 4 = 6 points. The average biopsy score was 2.27 ± 0.57 . The average number of significant rejection episodes per patient was 2.5 ± 1.8 .

Increased predisposition toward AR was recognized in patients who had more than 2 episodes of significant AR (20 individuals; 29%) and/or in patients with at least 1 episode of AR occurring more than 1 year after OHT (18 individuals; 26%).

Vessel abnormalities were assessed in EMB samples with an ISHLT rejection grade of 1B or less. EMB specimens with an ISHLT rejection grade of 2 or more and without arterioles were excluded from further investigation. From the 1071 EMB samples, we selected 517 (48%) samples fulfilling these criteria, in which vessel abnormalities and lipofuscin deposits were assessed. Hematoxylin and eosin staining was performed according to standard protocols. Additionally, selected slides were stained with Masson trichrome to assess interstitial fibrosis and myocytolysis. We assessed the following vessel abnormalities: presence of prominent endothelial cells, degeneration of the endothelium, thickening of the muscular layer, thickening of the vascular wall (basal membrane and muscle layer), presence of lymphocytes in the arteriolar wall without any other signs of rejection vasculitis, periarterial fibrosis, and an occluded arteriolar lumen (Figure E1). Additionally, the same biopsy slides were evaluated for the presence of lipofuscin (Figure E2). Lipofuscin deposits were visualized as bright yellow autofluorescent granules under ultraviolet light using an epifluorescence microscope.

The presence of particular vascular abnormalities and lipofuscin deposits in the selected set of 517 EMB samples was correlated with the grade of rejection (ISHLT grade 0–1B), using the *R* Spearman test. Biopsy specimens obtained 2 weeks after OHT and 12 and 36 months after OHT (or the closest time) were compared with the occurrence of the following:

- Any CAV confirmed by CAG, that is, the presence of critical and noncritical coronary stenosis observed in the main vessels, as well as in the primary and secondary branches, and/or features characteristic of distal vessel occlusion (slow passage of contrast through the vessels, lack of small vessels in the background for primary arteries and branches, significantly smaller than the expected diameter of the vessel lumen)
- Significant CAV, that is, the presence of hemodynamically significant coronary stenosis requiring coronary angioplasty, any critical stenosis ($\geq 90\%$ diameter stenosis) in the main coronary artery or the primary and secondary branch, myocardial infarction, or death caused by CAV)
- Death from all causes.

The first elective CAG took place 12 to 18 months after OHT. Consecutive CAGs were performed at least every 24 months.

EMB samples were reviewed by two experienced pathologists who were blinded to the CAG results, and each CAG was read by at least two invasive cardiologists who were not informed of the EMB results.

Continuous variables were presented as mean ± SD. Spearman coefficients and *P* values were calculated to estimate the correlation between the grade of AR and vascular abnormalities found in EMB samples. Odds ratios and 95% confidence intervals were calculated to assess the probability of developing any CAV, significant CAV, or death from any cause depending on the presence of classic risk factors of atherosclerosis and EMB findings. Kaplan–Meier curves were constructed to compare the time to CAV development in patients with and without lipofuscin deposits and particular vascular abnormalities. Significance values were calculated by log–rank tests (*P* > .1 was marked as NS). Follow-up was completed on January 31, 2006.

RESULTS

The correlations between the rejection grade and the presence versus absence of vascular abnormalities are listed in Table 1. Five of seven analyzed vascular features were found to be significantly related to cellular rejection: occurrence of enlarged endothelial cells, presence of single lymphocytes inside the arteriolar wall, occluded arteriolar lumen, punctiform endothelial vacuolization, and the presence of vascular muscle hypertrophy. There was no correlation between the presence of lipofuscin and rejection grade.

CAV was confirmed by CAG in 42 (62%) patients, CAV-related clinical events were observed in 23 (34%) patients, and death occurred in 35 (51%) patients. Analysis of odds ratios for classic risk factors of atherosclerosis, markers of high predisposition towards AR, particular vascular abnormalities, and the presence of lipofuscin in EMB samples revealed that none of these factors significantly increased the probability of any CAV or significant CAV development. The presence of periarteriolar fibrosis in the 3-year EMB increased the probability of death, and enlarged endothelial cell occurrence decreased the risk of death (Table E1).

Comparisons of the survival time after OHT without any CAV, with significant CAV, and death from any cause with particular vascular diseases in the 2-week, 12-month, and 36-month EMB samples are listed in Table 2. The only vascular abnormality observed in the 2-week EMB specimens predicting shorter time to the CAV-related clinical events was the presence of lymphocytes in the arterial wall. Another significant difference was found between patients with and without thickening of the vascular wall 12 months after OHT; the only 2 patients with this feature both had significant CAV before the end of the seventh year after OHT. None of the vascular abnormalities observed in the 2-week or 12-month EMB specimens predicted a shorter survival time, whereas a significant increase in survival time was found in patients without periarteriolar fibrosis in the 36-month EMB sample (Figure E3).

Lipofuscin was found in 23 (34%) patients in the 12-month EMB sample and in 30 (47%) patients in the 36-month EMB

TABLE 1. Correlations between the rejection grade and presence versus absence of vascular abnormalities (Spearman coefficients) based on an analysis of 517 EMB samples with an ISHLT rejection grade of 1B or less and arterioles suitable for analysis

Vascular abnormalities	R value, P value
Presence of prominent endothelial cells	R = 0.44, P < .001
Degeneration of endothelium	R = −0.19, P < .001
Thickening of muscle layer	R = −0.20, P < .001
Thickening of basal membrane and muscle layer	NS
Presence of lymphocytes in arteriolar wall	R = 0.44, P < .001
Periarteriolar fibrosis	NS
Occluded arteriolar lumen	R = 0.79, P < .001

EMB, Endomyocardial biopsy; ISHLT, International Society for Heart and Lung Transplantation; R, Spearman coefficient; NS, not significant.

sample. Of the 20 patients positive for lipofuscin in the 1-year EMB specimen for whom 36-month EMB samples were available for analysis, lipofuscin was observed in 15 (75%). Patients with lipofuscin in the 12-month EMB sample were characterized by a faster development of CAV (*P* < .08), significant CAV (*P* < .03), and survival (*P* < .07, log–rank test) (Figure 1). This relationship was not observed with lipofuscin positive samples in the 36-month EMB samples (Figure 2).

Lipofuscin was also present in the 2-week EMB samples of 2 patients; 12-month and 36-month EMB samples of these patients were lipofuscin negative.

DISCUSSION

The potential relationship between AR and CAV, detected both by angiography and by microvasculopathy in EMB samples, remains controversial. Some degree of vasculitis, which is typical of lymphocyte migration into the myocardium during AR, seems to be reversible. Its presence in EMB samples is not synonymous with the preclinical phase of CAV. For this reason, some studies analyze vascular abnormalities only in ISHLT grade 0 EMB specimens, and we do not disagree with this approach.¹⁰ However, the limitation of this study to patients with no signs of AR in the 1- and 3-year EMBs would exclude the majority of those enrolled from further investigation. We decided to exclude only EMB specimens with active myocytolysis of ISHLT grade 2 and higher. We confirmed a relationship between lower grades of AR and the majority of pathologic microvasculature features, suggesting that some of these may be reversible. This may be due to the lack of relationship between early microvasculopathy and late disease of epicardial coronary vessels. However, our results do agree with reports that revealed a relationship between microvasculopathy and a history of AR, even in EMB specimens without any signs of current active rejection, which shows that a potential bias from AR is not easily eliminated.¹⁰ On the other hand, we did not find any relationship between microvascular disease and epicardial coronary artery disease, even when only AR-independent features were taken into consideration.

TABLE 2. Comparison of the time free from the development of any CAV (confirmed by CAG), significant CAV (critical stenosis, myocardial infarction, need of percutaneous coronary intervention, or death caused by CAV), and death from all causes in patients with and without a particular vascular abnormality observed in the 2-week, 12-month, and 36-month EMB findings (log-rank test)

	Vascular abnormalities in EMB 37 ± 49 days after OHT				Vascular abnormalities in EMB 1.06 ± 0.28 y after OHT				Vascular abnormalities in EMB 4.53 ± 2.68 y after OHT			
	No. of pts (+/-)	P value to predict any CAV	P value to predict significant CAV	P value to predict death of any cause	No. of pts (+/-)	P value to predict any CAV	P value to predict significant CAV	P value to predict death of any cause	No. of pts (+/-)	P value to predict any CAV	P value to predict significant CAV	P value to predict death of any cause
Prominent endothelial cells	25/43	.37	.16	.26	39/29	.39	.17	.28	33/31	.20	.15	.16
Degeneration of endothelium	11/57	.76	.81	.96	38/30	.18	.94	.72	37/27	.48	.12	.37
Thickening of muscle layer	16/52	.35	.67	.60	13/55	.19	.82	.06	9/55	.93	.61	.58
Thickening of basal membrane and muscle layer	7/61	.55	.84	.47	2/66	.20	.04	.73	5/59	.91	.80	.89
Lymphocytes in arterial wall	4/64	.28	.04	.42	8/60	.56	.11	.33	14/50	.27	1.00	.37
Periarteriolar fibrosis	22/46	.67	.73	.97	38/30	.93	.67	.97	37/27	.09	.73	<.01
Diminished lumen	8/60	.33	.30	.95	6/62	.23	.74	.30	1/63	.19	.36	.31

CAV, Coronary vasculopathy; CAG, coronary angiography; EMB, endomyocardial biopsy; OHT, orthotopic heart transplantation.

We were unable to detect any correlation between the abnormalities in microvasculature analyzed in this study and epicardial CAV. This hypothetical relationship was absent not only 12 months after transplantation, but also when comparing EMB and CAG results 3 years after transplantation (ie, when epicardial lesions are not infrequent). Although correlating the EMB vascular changes with clinical CAV remains controversial,¹⁵ we agree with the reports that challenge the diagnostic value of EMB in predicting or confirming epicardial coronary artery disease.^{16,17} It is worth emphasizing that it is easier to show the relationship between microvasculopathy and AR, or even survival, than it is to correlate microvasculopathy with epicardial coronary disease.¹¹

None of the early (12-month) vascular diseases were predictive for decreased survival time, and the only feature discriminating patients with shorter survival times from those with increased survival was periarteriolar fibrosis observed in the 36-month EMB samples. This pathologic condition was not correlated with the degree of AR, and its role in the pathogenesis of transplanted heart failure should be investigated further.

Myocardial cellular senescence is accompanied by lipofuscin accumulation (aggregates of oxidized and cross-linked insoluble proteins). Lipofuscin formation depends on the oxidation of mitochondrial proteins produced by oxidative stress. As such, ischemic and rejection episodes may play an important role in lipofuscin deposition.¹⁸ Roffe¹⁹ identifies lipofuscin deposition, interstitial cardiac fibrosis, relative cardiocyte hypertrophy, and lower capillary density as indicative of age-related heart histology. The three former changes are most frequent in posttransplant heart biopsy tissue, and the decreased density of capillaries results from CAV. Lipofuscin deposits can also play a role in the regulation of cellular homeostasis by inducing apoptosis.²⁰ Thus, attempts to uncover links between CAV, the presence of lipofuscin, and the clinical course are promising avenues of research.

In contrast to vascular disease, lipofuscin deposition in the cytoplasm of cardiocytes observed 12 months after transplantation seems to be a good predictor of CAV development. This observation has interesting implications; particularly, since lipofuscin is a marker of aging, the mechanism of CAV development may include elements of accelerated aging occurring in the allograft during the first 12 months after transplantation, and rapid aging after the procedure may indicate development of CAV. Endothelial cell degradation is thought to be the critical stage for vasculopathy development—further disease is a result of the undesirable repair mechanisms involving myocyte migration and proliferation and resulting in neointima formation.⁴ We hypothesize that early cardiocyte aging could be related to the rapid endothelial degradation that provokes those repair processes. Why the first year after OHT is so important is a phenomenon that awaits explanation, but it has been shown that 1-year intimal growth is a marker of poor prognosis.^{21,22}

FIGURE 1. Comparison of the time free from the development of any angiographically confirmed CAV (A), significant CAV (B), and survival (C) in patients with and without lipofuscin deposits presence in cytoplasm of cardiocytes observed in the 12th-month EMB specimen. CAV, Cardiac allograft vasculopathy; EMB, endomyocardial biopsy; OHT, orthotopic heart transplantation; TxCAD, transplant coronary artery disease; MI, myocardial infarction; CAG, coronary angiography.

Notably, the same marker of aging observed 3 years after OHT seems to have no predictive value for CAV development and death. Lipofuscin deposition is more common at

this time (in almost half of the patients, compared with only one third of OHT recipients at the end of the first year after transplantation), but only 75% of the individuals

FIGURE 2. Comparison of the time free from the development of any angiographically confirmed CAV (A), significant CAV (B), and survival (C) in patients with and without lipofuscin deposits present in the cytoplasm of cardiocytes observed in the 36th month EMB specimen. CAV, Cardiac allograft vasculopathy; EMB, endomyocardial biopsy; OHT, orthotopic heart transplantation; TxCAD, transplant coronary artery disease; MI, myocardial infarction; CAG, coronary angiography; PCI, percutaneous coronary intervention; NS, not significant.

with lipofuscin in the 1-year EMB results remained positive 2 years later. Additionally, patients with lipofuscin present in EMB samples obtained 2 weeks after OHT showed an ab-

sence of lipofuscin at 12 and 36 months after OHT. Specifically, 25% of patients with lipofuscin-positive EMBs 12 months after OHT did not show this marker 2 years later.

For the purpose of this study, it was crucial to use a method that allowed the examination of the same EMB slides that were used to assess a rejection grade at a time of procurement, inasmuch as there was no any additional tissue material that could be used for more specific identification of lipofuscin. A method of identifying lipofuscin using its autofluorescent properties is widely accepted and used in many areas of histologic investigation.

The issue of lipofuscin elimination itself seems to be very interesting. Early death of cells with lipofuscin deposits is not the only mechanism for clearance; a lipofuscin elimination mechanism was described in retinal pigment epithelium in 1999,²³ which implicated protease inhibition in lipofuscin disposal. Although we do not as yet have direct evidence for enhanced lysosomal activity in postmitotic transplanted cardiomyocytes, we postulate that the intracellular metabolic pathway activity would be increased during myocardial remodeling.

Our study is the first attempt to correlate lipofuscin deposition in cardiomyocytes with CAV; the only previous publication confirming the presence of lipofuscin in transplanted heart myocytes does not examine this process in the context of CAV.²⁴

Two major limitations of this study are its retrospective nature and the low number of observed subjects, which may bias statistical testing. The necessity to use EMB slides with rejection grades higher than 0, according to the ISHLT, was mentioned earlier. Intravascular coronary ultrasound was not used in the majority of patients, so it was not possible to use these results to standardize a diagnosis of CAV. Additionally, the category of any CAV was biased by the lack of routine CAG performed in the donor or immediately after the OHT to exclude coronary disease in the donor at the time of the investigation. All of these limitations should be taken into account when planning larger prospective trials to confirm the results of this study.

References

1. Taylor DO, Edwards LB, Boucek MM, Trulock EP, Aurora P, Christie J, et al. Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult heart transplant report—2007. *J Heart Lung Transplant*. 2007;26:769-81.
2. Wong C, Ganz P, Miller L, Kobashigawa J, Schwarzkopf A, Valantine von Kaepfer H, et al. Role of vascular remodeling in the pathogenesis of early transplant coronary artery disease: a multicenter prospective intravascular ultrasound study. *J Heart Lung Transplant*. 2001;20:385-92.
3. Tsutsui H, Ziada KM, Schoenhagen P, Iyisoy A, Magyar WA, Crowe TD, et al. Lumen loss in transplant coronary artery disease is a biphasic process involving early intimal thickening and late constrictive remodeling: results from a 5-year serial intravascular ultrasound study. *Circulation*. 2001;104:653-7.
4. Mehra MR. Contemporary concepts in prevention and treatment of cardiac allograft vasculopathy. *Am J Transplant*. 2006;6:1248-56.
5. Pethig K, Klauss V, Heublein B, Mudra H, Westphal A, Weber C, et al. Progression of cardiac allograft vascular disease as assessed by serial intravascular ultrasound: correlation to immunological and non-immunological risk factors. *Heart*. 2000;84:494-8.
6. Valantine H, Rickenbacker P, Kemna M, Hunt S, Chen YD, Reaven G, et al. Metabolic abnormalities characteristic of dysmetabolic syndrome predict the development of transplant coronary artery disease: a prospective study. *Circulation*. 2001;103:2144-52.
7. Kobashigawa JA, Miller L, Yeung A, Hauptman P, Ventura H, Wilensky R, et al. Does acute rejection correlate with the development of transplant coronary artery disease? A multicenter study using intravascular ultrasound. Sandoz/CVIS Investigators. *J Heart Lung Transplant*. 1995;14:S221-6.
8. Jimenez J, Kapadia SR, Yamani MH, Platt L, Hobbs RE, Rincon G, et al. Cellular rejection and rate of progression of transplant vasculopathy: a 3-year serial intravascular ultrasound study. *J Heart Lung Transplant*. 2001;20:393-8. Erratum in: *J Heart Lung Transplant*. 2001;20:803.
9. Yamani MH, Yousufuddin M, Starling RC, Tuzcu M, Ratliff NB, Cook DJ, et al. Does acute cellular rejection correlate with cardiac allograft vasculopathy? *J Heart Lung Transplant*. 2004;23:272-6.
10. Hiemann NE, Wellnhofer E, Hetzer R, Meyer R. Small vessel disease after heart transplantation: impact of immunologic and nonimmunologic risk factors. *Transpl Int*. 2005;18:908-14.
11. Hiemann NE, Wellnhofer E, Knosalla C, Lehmkühl HB, Stein J, Hetzer R, et al. Prognostic impact of microvasculopathy on survival after heart transplantation: evidence from 9713 endomyocardial biopsies. *Circulation*. 2007;116:1274-82.
12. Szweida PA, Camouse M, Lundberg KC, Oberley TD, Szweida LI. Aging, lipofuscin formation, and free radical-mediated inhibition of cellular proteolytic systems. *Ageing Res Rev*. 2003;2:383-405.
13. Jung T, Bader N, Grune T. Lipofuscin: formation, distribution, and metabolic consequences. *Ann N Y Acad Sci*. 2007;1119:97-111.
14. Terman A, Brunk UT. Autophagy in cardiac myocyte homeostasis, aging, and pathology. *Cardiovasc Res*. 2005;68:355-65.
15. Lozano MD. Microvascular coronary arterial vasculopathy—predictive value of endomyocardial biopsy. *Z Kardiol*. 2000;89:IX/54-7.
16. Clausell N, Butany J, Molossi S, Lonn E, Gladstone P, Rabinovitch M, et al. Abnormalities in intramyocardial arteries detected in cardiac transplant biopsy specimens and lack of correlation with abnormal intracoronary ultrasound or endothelial dysfunction in large epicardial coronary arteries. *J Am Coll Cardiol*. 1995;26:110-9.
17. Armstrong AT, Strauch AR, Kardan A, Starling RC. Morphometric and immunocytochemical analysis of coronary arterioles in human transplanted hearts. *J Heart Lung Transplant*. 1996;15:818-26.
18. Lefensky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia reperfusion, aging, and heart failure. *J Mol Cell Cardiol*. 2001;33:1065-89.
19. Roffe C. Ageing of the heart. *Br J Biomed Sci*. 1998;55:136-48.
20. Powell SR, Wang O, Divald A, Teichberg S, Haridas V, McCloskey TW, et al. Aggregates of oxidized proteins (lipofuscin) induce apoptosis through proteasome inhibition and dysregulation of proapoptotic proteins. *Free Radic Biol Med*. 2005;38:1093-101.
21. Kobashigawa JA, Tobis JM, Starling RC, Tuzcu EM, Smith AL, Valantine HA, et al. Multicenter intravascular ultrasound validation study among heart transplant recipients: outcomes after five years. *J Am Coll Cardiol*. 2005;45:1532-7.
22. Tuzcu EM, Kapadia SR, Sachar R, Ziada KM, Crowe TD, Feng J, et al. Intravascular ultrasound evidence of angiographically silent progression in coronary atherosclerosis predicts long-term morbidity and mortality after cardiac transplantation. *J Am Coll Cardiol*. 2005;45:1538-42.
23. Katz ML, Rice LM, Gao CL. Reversible accumulation of lipofuscin-like inclusions in the retinal pigment epithelium. *Invest Ophthalmol Vis Sci*. 1999;40:175-81.
24. Seichertová A, Urbanová D. Electron microscopic and light microscopic investigation of endomyocardial biopsies in the transplanted heart. *Funct Dev Morphol*. 1993;3:11-7.

FIGURE E1. Analyzed vascular abnormalities: A, Myocardial arteriole—endothelial cells (*red arrow*), muscular layer (*black arrow*), thin intima (*green arrow*). B, Myocardial arteriole—enlarged endothelial cells (*red arrows*) and light and focal vacuolation (*black arrow*). C, Myocardial arteriole—focal intimal thickening (*red arrow*) and prominent endothelial cells (*black arrow*). D, Myocardial arteriole—focal muscular hypertrophy (*green arrow*), recent fibrolipoid plaque (*red arrow*), lipid-containing cells (*black arrow*), and slightly enlarged endothelial cells (*white arrow*). E, Myocardial arteriole—focal intimal thickening and hyalinized arteriolar wall (*black arrow*). F, Myocardial arteriole—periarteriolar fibrosis. G, Myocardial arteriole—periarteriolar fibrosis collagen stained green. H, Myocardial arteriole—vacuolization: hydropic degeneration (*black arrows*). I, Myocardial arteriole—lymphocytes inside the arteriolar wall (*green arrow*).

FIGURE E2. Lipofuscin in the cytoplasm of cardiocytes, assessed under fluorescent microscope (lipofuscin in *yellow*).

FIGURE E3. Comparison of the survival time in patients with and without periarteriolar fibrosis observed in the 36th-month EMB specimen. EMB, Endomyocardial biopsy; OHT, orthotopic heart transplantation.

TABLE E1. Probability of any CAV (confirmed by coronary angiography), significant CAV (critical stenosis, myocardial infarction, need of percutaneous coronary intervention, or death caused by CAV), and death from all causes in patients with particular risk factors, including classic risk factors of ischemic heart disease, high predisposition toward AR, and 12- and 36-month EMB findings (odds ratios, 95% confidence intervals, and *P* values)

Risk factor	n (+)	n (−)	Probability of any CAV			Probability of significant CAV			Probability of death		
			OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Clinical risk factors											
Sex (male)	63	5	2.66	0.41–17.15	NS	2.42	0.25–23.01	NS	1.65	0.26–10.56	NS
Age (≥40 y)	47	21	1.28	0.43–3.81	NS	0.78	0.26–2.31	NS	0.95	0.34–2.66	NS
Age (≥50 y)	21	47	0.55	0.19–1.58	NS	1.12	0.38–3.26	NS	1.39	0.49–3.92	NS
Ischemic cardiomyopathy	35	33	2.12	0.77–5.83	NS	2.67	0.94–7.59	NS	1.26	0.49–3.27	NS
BMI ≥ 25	34	34	0.88	0.32–2.38	NS	1.30	0.48–3.55	NS	0.55	0.21–1.45	NS
Diabetes mellitus	11	57	1.08	0.28–4.14	NS	1.58	0.43–5.85	NS	0.75	0.21–2.74	NS
Arterial hypertension	30	38	0.85	0.31–2.30	NS	1.02	0.37–2.80	NS	1.85	0.70–4.89	NS
Hyperlipidemia	45	23	1.61	0.57–4.58	NS	1.85	0.61–5.63	NS	1.63	0.59–4.47	NS
More than 2 episodes of significant AR	20	48	1.64	0.53–5.04	NS	0.92	0.31–2.75	NS	0.69	0.24–1.97	NS
Significant AR ≥ 1 y after OHT	18	50	1.86	0.57–6.05	NS	2.20	0.73–6.66	NS	0.36	0.12–1.12	NS
Abnormalities in 2-week EMB											
Prominent endothelial cells	25	43	0.75	0.27–2.09	NS	0.57	0.20–1.66	NS	0.80	0.30–2.15	NS
Degeneration of endothelium	11	57	1.47	0.34–6.28	NS	0.68	0.16–2.92	NS	0.75	0.21–2.74	NS
Thickening of muscle layer	16	52	0.89	0.27–2.89	NS	1.75	0.54–5.64	NS	2.57	0.78–8.43	NS
Thickening of basal membrane and muscle layer	7	61	0.45	0.09–2.18	NS	1.36	0.28–6.65	NS	0.68	0.14–3.30	NS
Lymphocytes inside arterial wall	4	64	1.89	0.19–19.28	NS	5.86	0.57–59.81	NS	—	—	NS
Periarteriolar fibrosis	22	46	0.88	0.30–2.58	NS	0.92	0.31–2.75	NS	0.70	0.25–1.94	NS
Diminished lumen	8	60	0.79	0.16–3.88	NS	0.67	0.12–3.77	NS	1.67	0.37–7.61	NS
Lipofuscin presence	2	66	—	—	NS	1.74	0.10–29.15	NS	—	—	NS
Abnormalities in 1-year EMB											
Prominent endothelial cells	39	29	0.55	0.19–1.54	NS	0.42	0.15–1.18	.0977	0.37	0.14–0.99	.0457
Degeneration of endothelium	38	30	1.69	0.62–4.62	NS	0.89	0.32–2.43	NS	0.69	0.26–1.80	NS
Thickening of muscle layer	13	55	0.65	0.19–2.22	NS	1.67	0.49–5.70	NS	0.52	0.15–1.79	NS
Thickening of basal membrane and muscle layer	2	66	—	—	NS	—	—	NS	0.94	0.06–15.69	NS
Lymphocytes inside arterial wall	8	60	0.57	0.13–2.51	NS	0.22	0.03–1.89	NS	0.27	0.05–1.46	NS
Periarteriolar fibrosis	38	30	1.00	0.37–2.73	NS	0.68	0.25–1.87	NS	1.11	0.43–2.90	NS
Diminished lumen	6	62	0.58	0.11–3.12	NS	0.86	0.15–5.10	NS	0.44	0.07–2.58	NS
Lipofuscin presence	23	45	1.48	0.50–4.37	NS	2.38	0.83–6.86	NS	1.78	0.64–4.94	NS
Abnormalities in 3-year EMB											
Prominent endothelial cells	33	31	0.71	0.25–2.00	NS	0.53	0.18–1.54	NS	0.47	0.17–1.27	NS
Degeneration of endothelium	37	27	0.83	0.29–2.35	NS	0.47	0.16–1.38	NS	0.79	0.29–2.13	NS
Thickening of muscle layer	9	55	0.76	0.18–3.16	NS	0.51	0.10–2.71	NS	1.39	0.34–5.75	NS
Thickening of basal membrane and muscle layer	5	59	0.94	0.15–6.10	NS	1.33	0.21–8.67	NS	1.66	0.26–10.68	NS
Lymphocytes inside arterial wall	14	50	0.38	0.11–1.27	NS	0.73	0.20–2.68	NS	0.34	0.09–1.23	NS
Periarteriolar fibrosis	37	27	2.69	0.94–7.71	.0621	1.04	0.36–3.02	NS	5.27	1.77–15.75	.0047
Diminished lumen	1	63	—	—	NS	—	—	NS	—	—	NS
Lipofuscin presence	30	34	1.56	0.55–4.36	NS	0.95	0.33–2.74	NS	0.88	0.33–2.34	NS

CAV, Coronary vasculopathy; AR, acute rejection; OHT, orthotopic heart transplantation; EMB, endomyocardial biopsy; BMI, body mass index; NS, not significant; OR, odds ratio; CI, confidence interval.