Can C4d Immunostaining on Endomyocardial Biopsies Be Considered a Prognostic Biomarker in Heart Transplant Recipients?

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Background. The aim of this study was to assess the significance of positive C4d capillary immunostaining of endomyocardial biopsies and its correlation to clinical outcome in adult heart transplant recipients.
Methods. Nine hundred eighty-five endomyocardial biopsies from 107 heart transplant recipients were evaluated. Immunostaining for detection of intragraft C4d capillary deposition was performed on paraffin-embedded tissue using anti-human C4d polyclonal antibody.
Results. Positive staining of C4d was present in 36 patients (34%) and antibody-mediated rejection in eight patients (7%). The patients were subdivided into four groups on the basis of their C4d, circulating antidonor antibodies (donor-specific antibodies [DSAs]), and graft function: group 1=C4d positive, DSA negative, and no graft dysfunction; group 2=C4d positive, DSA positive, and no graft dysfunction; group 3=C4d positive, DSA positive, and signs of graft dysfunction, and group 0 (control)=all negative. An higher mortality risk was found in C4d-positive patients, when compared with negative ones (unadjusted hazard ratios: group 1: 18, group 2: 61, and group 3: 32-fold risk; $P<0.0001$).
Conclusions. Antibody-mediated rejection is a complex and ongoing phenomenon with different phenotypic features. C4d positive predicts worse prognosis. C4d negative and DSA can be used as early mortality predictors in patients without signs of graft dysfunction.

Keywords: AMR, C4d complement deposition, Transplant, Endomyocardial biopsy, Immunohistochemistry, CAV.

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There is an increasing interest in the role of humoral immune mechanisms in cardiac allograft rejection. Studies performed during the past decade have indicated that humoral rejection of solid organ transplantation is associated with C4d linear deposits along the graft capillaries (*1–4*). Positivity of C4d staining has been considered an independent predictor of kidney graft dysfunction and a reliable specific marker for antibody-dependent graft injury (*5–11*). Capillary deposition of complement C4d is considered an important tissue marker of humoral rejection in heart transplantation as well (*12*, *13*). In accordance with the International Society for Heart and Lung Transplantation (ISHLT) recommendations, diagnosis of antibody-mediated cardiac allograft rejection (antibody-mediated rejection [AMR]) implies clinical evidence of graft dysfunction, histologic evidence of acute capillary injury, and immunopathologic evidence for antibody-mediated injury as C4d capillary positivity on endomyocardial biopsies (EMBs) (*14*). Nevertheless, some studies have reported that not all patients have allograft dysfunction after kidney (*15*, *16*) or heart transplantation, despite C4d deposition, (*17*), and thus, the term "asymptomatic AMR" was introduced. It has been suggested that complement regulatory proteins can successfully terminate the complement cascade after activation in renal and heart transplants in the attempt to achieve a state of accommodation (*18–*

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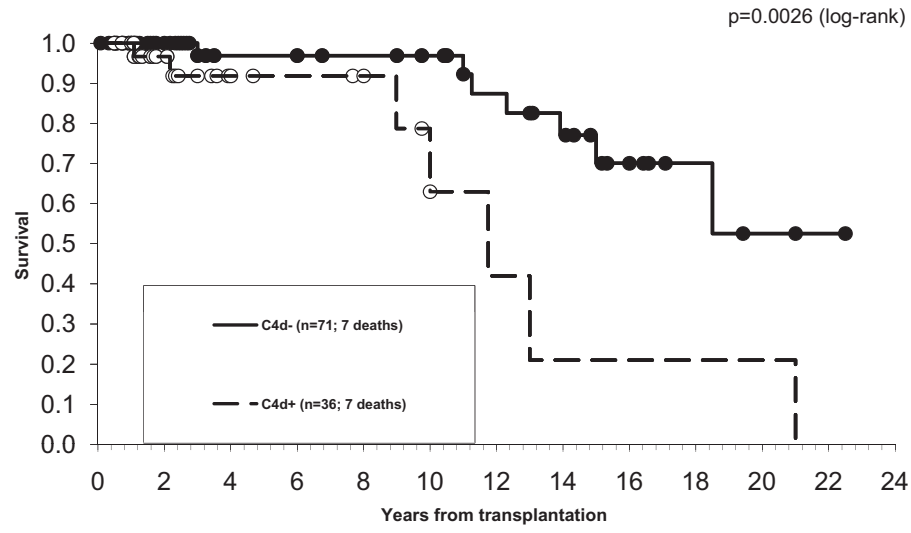

FIGURE 1. Survival of C4d-positive group compared with C4d-negative control group (Kaplan-Meier test).

20). Although it is unclear whether complement deposits in asymptomatic patients reflect accommodation or subclinical undetectable AMR, there is even less evidence of their short- or long-term consequences in asymptomatic patients.

AMR has been detected at histology in patients with normal cardiac function and no symptoms of heart failure (asymptomatic) who are not generally prescribed treatment. Little information is presently available concerning the significance of capillary positivity on EMB specimens of asymptomatic patients (asymptomatic AMR [AsAMR]) (*21*).

Although few studies have shown that capillary deposition of complement split product C4d is associated with the development of cardiac allograft vasculopathy (CAV) detected by intravascular ultrasound, immunohistochemical evaluation of serial cardiac allograft biopsies seems to identify the patients at risk (*22–24*). The aim of this study was to assess the diagnostic and prognostic significance of C4d-positive capillary staining detected by the immunoperoxidase methodology performed on paraffin-embedded tissue sections in the heart transplant recipients (HTx).

RESULTS

Of the 985 consecutive cardiac allograft biopsies performed, 56 (5.7%) from 36 of 107 patients (34%) were found to have C4d deposits. Fourteen (39%) of these were also positive to donor-specific antibodies (DSAs). Five patients were found to be positive to DSA within the first year of transplantation. Of the 14 positive to C4d immunostaining and DSA, eight (57%) demonstrated signs of allograft dysfunction. On the basis of our criteria, 22 of the patients fell into group 1, six into group 2, eight into group 3, and 71 into the control group (group 0). Groups 1 and 2 represented the asymptomatic patients and group 3 the symptomatic ones.

Patients in group 1 became positive to C4d after transplantation after a median time of 2.2 months (range: 0.37–121.40 months), those in group 2 after 0.70 months (range: 0.43–25.17 months), and those in group 3 after 112.42 months (range: 14.10–251.87 months) (see **Table, Supplemental Digital Content 1,** http://links.lww.com/TP/A231).

C4d and Survival

Fourteen patients died during the follow-up at a median of 2.7 years after transplantation (range: 1–22.5 years). The mortality (see **Table, Supplemental Digital Content 2,** http://links.lww.com/TP/A232) was higher in group 2 with respect to group 3, but death occurred earlier after C4d positive in group 3. The 22 patients in group 1 (61% of total C4d positive) showed an 18-fold higher risk compared with the C4d-negative patients (95% confidence interval [CI]: 1.960–160.022). The six patients in group 2 had a 61-fold higher risk (95% CI: 3.399–1110.360). The eight patients in group 3 had a 32-fold higher risk (95% CI: 5.884–179.432), overall $P<0.0001$ (see **Table, Supplemental Digital Content 3,** http://links.lww.com/TP/A233). Overall, the C4d-positive patients showed a statistically significant reduction in survival compared with the C4d-negative patients (Fig. 1), and this observation was preserved in the three different groups (see **Figure, Supplemental Digital Content 4,** http://links.lww.com/TP/A234). When the asymptomatic (groups 1 and 2) and symptomatic patients (group 3) were compared with the control group, an 18- and 26-fold increase mortality risk was observed, respectively (see **Table, Supplemental Digital Content 5,** http://links.lww.com/TP/A235).

No differences were found between the C4d-positive and negative groups in patients' ages, sex, number of transfusions, pregnancy, and time of ischemia during transplantation (Table 1). Of the 107 patients included in this study, six received left ventricular assist device (6%), three were positive and three were negative to C4d. One case had humoral rejection and recurrence of C4d (2.5%), and two patients had asymptomatic AsAMR (7.0%). Pretransplant panel reactive antibody (PRA) test values were available for all 36 C4d-positive patients and for 75% of the control group. There were three PRA-positive patients in group 3, none in group 2, and two in group 1.

Histologic Features

All the EMBs positive to C4d were evaluated for the histologic parameters of AMR. The results (Table 2) showed that morphologic features were present in only a limited

TABLE 1. Univariate analysis of clinical and pathologic variables to identify risk factors for the development of C4d positivity

Clinical variables	Group 0 (n=71)	Group 1 (n=22)	Group 2 (n=6)	Group 3 (n=8)	*P*
Sex: M, n (%)	57 (80.3)	15 (68.2)	5 (83.3)	8 (100.0)	0.2888[a]
Recipient age (yr), median (min–max)	56 (17–73)	49.5 (19–69)	52 (34–60)	52 (36–59)	0.5347[b]
Transplant indications, n (%)					0.4692[a]
Cardiomyopathies	36 (50.7)	9 (40.9)	3 (50.0)	2 (25.0)	
Ischemic cardiomyopathies	23 (32.4)	6 (27.3)	2 (33.3)	4 (50.0)	
Valvular cardiomypathies	3 (4.2)	1 (4.6)	1 (16.7)	0 (0.0)	
Congenital heart disease	2 (2.8)	3 (13.6)	0 (0.0)	0 (0.0)	
Others	7 (9.9)	3 (13.6)	0 (0.0)	2 (25.0)	
Transfusions, n (%)	7 (12.5)	6 (28.6)	2 (33.3)	1 (12.5)	0.2008[a]
Mismatch, n (%)	38 (62.3)	11 (50.0)	5 (83.3)	6 (75.0)	0.4351[a]
Donor age (yr), median (min–max)	37 (17–64)	35 (15–66)	46 (15–58)	32.5 (17–59)	0.8326[b]
Incompatibility A, n (%)	35 (58.3)	14 (63.6)	6 (100.0)	6 (62.5)	0.2609[a]
Incompatibility B, n (%)	36 (60.0)	16 (72.7)	6 (100.0)	5 (62.5)	0.2080[a]
Incompatibility DR, n (%)	34 (56.7)	15 (68.2)	6 (100.0)	4 (50.0)	0.1545[a]
Donor cause of death, n (%)					0.8801[a]
Trauma	30 (51.7)	11 (52.4)	4 (66.7)	5 (62.5)	
Cerebrovascular disease	23 (39.7)	10 (47.6)	2 (33.3)	3 (37.5)	
Other	5 (8.6)	0 (0.0)	0 (0.0)	0 (0.0)	
Viruses, n (%)	26 (41.3)	11 (57.9)	4 (66.7)	5 (62.5)	0.3333[a]
Pregnancies (only in women), n (%)	10 (83.3)	6 (100.0)	1 (100.0)	0 (0.0)	0.5789[a]
Time of cold ischemia minutes median (min–max)	162 (0–300)	193.5 (0–300)	160 (110–180)	120 (120–240)	0.5467[b]
Rejection score median (min–max)	0.82 (0.00–2.68)	0.94 (0.13–1.92)	0.81 (0.57–1.54)	1.13 (0.0–1.56)	0.8750[b]
Rejection score severe median (min–max)	0.24 (0.00–1.05)	0.28 (0.00–1.61)	0.38 (0.29–1.17)	0.19 (0.00–0.730)	0.1523[b]

None of the considered variables were statistically significant.
[a] Fisher exact test.
[b] Kruskal-Wallis test.

TABLE 2. Histologic findings

Major histologic characteristics for AMR diagnosis	Group 1 (22 patients) n (%)	Group 2 (6 patients) n (%)	Group 3 (8 patients) n (%)
Endothelial swelling	25 (83)	5 (71)	8 (100)
Endothelial denudation	11 (37)	4 (57)	1 (12)
Neutrophils in capillaries	6 (20)	3 (43)	2 (25)
Macrophages in capillaries	14 (47)	3 (43)	3 (37)
Interstitial edema	23 (77)	6 (86)	4 (50)
Hemorrhage	7 (23)	1 (14)	1 (12)

AMR, antibody-mediated rejection.

number and cannot be used to select patients for immunistochemistry staining. They were also unable to discriminate between the different groups and the clinical outcome. Prominent accumulation of intracapillary macrophages was not observed in all patients with AMR (only 37%), and the morphologic features were found to be heterogeneous in the three groups (Fig. 2).

Recurrence of C4d Positivity on EMB

In the positive C4d patients, there were nine (25%) with recurrent C4d positivity on EMB, seven of them had multiple rejection episodes (78%) characterized by at least one negative between two positive biopsies; one of them (1/8 of group 3, 12.5%) was diagnosed with AMR 7 years after heart transplantation and was treated with plasmapheresis and rituximab. The other eight became positive within the first year after transplantation (median: 1.58 months, range: 0.47–8.07 months). No clinical data were statistically significant in this group (Table 3). Six of the nine (67%) patients presented anti-human leukocyte antigens antibodies, and among them, three had DSA and three no DSA. Survival was worse in the C4d-positive patients with recurrence with respect to the controls ($P=0.0662$) (see **Figure, Supplemental Digital Content 6,** http://links.lww.com/TP/A236).

Outcome of Patients With AMR

AMR diagnosis was confirmed in eight patients on the basis of the ISHLT criteria. AMR occurred at a median of 9 years (range: 1 month–21 years) after heart transplantation. Two patients had recurrence of C4d positivity and DSA detection after antibody-mediated rejection therapy. Two of the eight patients had received a left ventricular assist device before heart transplantation, and four had a previous diagnosis of Epstein-Barr virus-related posttransplant lymphoproliferative disease. All of them were on a chronic immunosuppressive regimen with cyclosporine, cyclosporine plus everolimus (five of eight, 62%), cyclosporine plus mycophenolic acid (three patients), and three patients were on steroids. Six (66%) of the patients with AMR were treated with

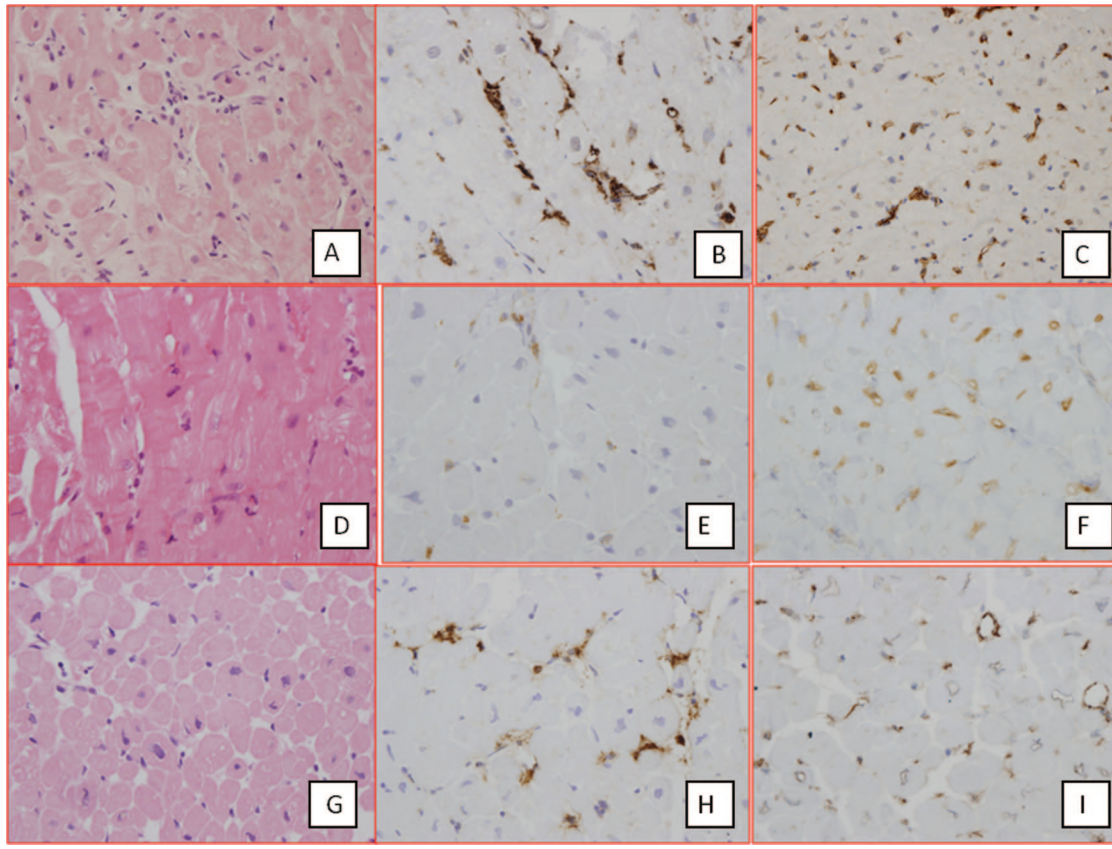

FIGURE 2. Examples of the immunopathologic features of the different studied groups. A 48-year-old man with AMR: (A) vascular infiltrate (ACR grades 1A, 1R), interstitial edema, and endothelial swelling (hematoxylin-eosin [H&E], original magnification ×320); (B) CD68 IHC staining with intracapillary macrophages; and (C) intense linear C4d staining of capillary endothelium with a diffuse pattern and strong intensity. A 60-year-old man of group 2: (D) scanty perivascular inflammatory infiltrate (H&E, original magnification ×320, ACR grade 0); (E) some macrophages in capillaries (CD68, original magnification ×320); and (F) C4d staining of capillary endothelium with diffuse pattern and strong intensity (original magnification ×320). A 49-year-old man of group 1: (G) negative for ACR and no signs suggestive for AMR (H&E, original magnification 320×); (H) some intracapillaries macrophages (CD68, original magnification ×320); and (I) C4d staining with a diffuse pattern and moderate intensity (original magnification ×320). AMR, antibody-mediated rejection; IHC, immunohistochemistry; ACR, acute cellular rejection.

rituximab and plasmapheresis, one patient received only plasmapheresis, and two patients did not received a specific treatment because of the contraindications.

Of the four patients (33%) who died as a result of AMR, one had never received rituximab or plasmapheresis because of their contraindications. Another patient, transplanted 21 years earlier, had been treated with three cycles of plasmapheresis. Ejection fraction ameliorated after the second cycle, but the patient died suddenly. The other two patients showed improvement after plasmapheresis and rituximab, but they died some time later, one due to posttransplant lymphoproliferative disorder and the other due to AMR.

C4d and CAV

Coronary angiography results were available for 59 of 71 C4d-negative patients (83%) and 30 of 36 C4d-positive patients (83%). CAV developed at a median of 1.37 years (range: 0.04–20.7 years) in the C4d-positive patients. No differences in angiographically detectable CAV were found in the C4d-positive patients (7/30) with respect to the C4d-negative patients (16/59). No statistically significance was found with respect to CAV when comparing the four groups (P=0.5697). Two patients (25%) in group 3 (AMR) developed CAV, none did in group 2, and five (31%) did in group 1.

C4d and Acute Cellular Rejection

Absence of acute cellular rejection (ACR) was observed in 24 (24/56, 43%) of the (56/985) C4d positive EMBs. Grade 1R (focal ACR) was found in 26 (26/56, 46%), grade 2R (moderate cellular rejection) was detected in five (9%), and grade 3R (severe cellular rejection) was found in one (2%). There were no differences in rejection score and severe rejection score in the four groups (Table 1). Nonetheless, patients with recurrent C4d positivity had a more severe rejection score with respect to the C4d-negative patients (median: 0.63, range=0.00–1.17 vs. median: 0.24, range=0.00–1.05, respectively; P=0.034).

DISCUSSION

Our findings indicate that C4d staining performed on a routine basis after heart transplantation on paraffin-embedded

TABLE 3. Univariate analysis of clinical variables to identify risk factors for development of C4d recurrence defined as more than one EMB C4d positivity

Clinical variables	C4d negative (n=71)	Recurrence C4d positive (n=9)	*P*
Sex: M, n (%)	57 (80.3)	7 (77.8)	1.0000[a]
Recipient age (yr), median (min-max)	56 (17–73)	49 (34–68)	0.3371[a]
Transplant indications n (%)			0.0488[a]
Cardiomyopathies	36 (50.7)	2 (22.2)	
Ischemic cardiomyopathies	23 (32.4)	2 (22.2)	
Valvular cardiomypathies	3 (4.2)	2 (22.2)	
Congenital heart disease	2 (2.8)	1 (11.1)	
Others	7 (9.9)	2 (22.2)	
Transfusions n (%)	7 (12.5)	3 (33.3)	0.1345[a]
Mismatch, n (%)	38 (63.3)	5 (55.6)	0.7258[a]
Donor age (yr), median (min–max)	37 (17–64)	35 (23–64)	0.7788[a]
Incompatibility A, n (%)	35 (58.3)	8 (88.9)	0.1383[a]
Incompatibility B, n (%)	36 (60.0)	8 (88.9)	0.1410[a]
Incompatibility DR, n (%)	34 (56.7)	8 (88.9)	0.0793[a]
Donor cause of death, n (%)			0.8682[a]
Trauma	30 (51.7)	6 (66.7)	
Cerebrovascular disease	23 (39.7)	3 (33.3)	
Other	5 (8.6)	0 (0.0)	
Viruses, n (%)	26 (41.3)	6 (75.0)	0.1285[a]
Pregnancies, n (%)	10 (83.3)	2 (100.0)	1.0000[a]
Time of cold ischemia minutes (min–max)	162 (0–300)	150 (96–300)	0.7986[b]
Rejection score median (min–max)	0.82 (0.00–2.68)	1.25 (0.42–1.59)	0.3223[b]
Rejection score severe median (min–max)	0.24 (0.00–1.05)	0.63 (0.00–1.17)	0.0345[b]

[a] Fisher exact test.
[b] Kruskal-Wallis test.
EMB, endomyocardial biopsy.

tissue sections can predict outcome in HTx and supporting the utility of C4d stain on routine base. Moreover, categorizing patients into four groups on the basis of their C4d, DSA, and graft function profile stratify the mortality risk (*25*). Our results showing that group 3 had half the mortality risk of group 2 (32- vs. 61-fold increase) is only apparently contradictory. Group 3 had the longest time interval between heart transplant and EMB-C4d positivity but the shortest survival time between C4d positive and death. Early acute versus late acute of chronic rejection episodes, humoral or cellular humoral, might represent different pathogenic situation with different prognosis, virus infections, particularly cardiotropic virus, immunosuppression, lymphoproliferative disorders, solid neoplasia, might producing "injury" with antigen modifications and, consequently, antibody formation. Injury produced by the activation of the complement cascade on the endothelium requires time to act and to dissolve the equilibrium produced by the regulatory mechanism (CD55 and CD59 proteins) for blockage of complement cascade (*17*), but when dysfunction appears, the patients' outcome worsens. Group 1 could represent those patients in whom DSA, although present, are undetected for two possible reasons: (1) the insensitivity of antibody detection methods and (2) sequestration of low levels of DSA in the graft (*26*).

Intragraft deposition of C4d complement split fragments could, then, be regarded as (1) a subclinical form of AMR incapable of producing clinically relevant graft dysfunction but acting over a long period of time as an immunologic noxa contributing to allograft vasculopathy or (2) accommodation as acquired resistance to pathologic effects of graft-specific antibodies and complement fixation (*27–29*). In a recent article, Rodriguez and coworkers (*17*, *30–34*) hypothesized that the complement cascade could be inhibited by the presence of regulatory proteins CD55 and CD59 capable of halting the activation of complement cascade. Our findings indicate that over time, the graft's ability to inhibit or neutralize the complement cascade activation on endothelial cells could decrease leading to graft dysfunction.

Our results are at difference with those of Wu et al. (*21*), the first to assess patients with AsAMR and to compare them with treated AMR, who reported no differences in fact between asymptomatic (our groups 1 and 2), treated AMR (our group 3), and controls (our group 0) in their 5-year survival rate. They did not assess the presence of DSA in their population of asymptomatic patients. Our study, in which DSA was assessed and the asymptomatic patients were divided into DSA-positive and -negative groups, showed that detection of DSA is a negative prognostic marker indicative of worse outcome.

Clinical data considered were unable to differentiate between the symptomatic and asymptomatic patients and between both of them and the controls. No factors, such as positive PRA with impact of sensitization, were found not significant. We cannot exclude that this could be ascribe to

low percentage of PRA before transplant which is low compared to majority of other studies of HTx (10%–20%) reasonable explanation the high proportions of male (80%) included in this study. Patients with more than one EMB specimen positivity for C4d (25% of the C4d-positive patients) had a worse survival rate and more severe rejection scores. These results are compatible with the hypothesis that serial occurrences of C4d positivity are indicative of repetitive episodes of endothelial injury (*35*). Our data seem to parallel those of Hammond et al. (*36*) who found that repetitive episodes of AMR increase the risk of cardiovascular mortality with an incremental risk of 8% for each episode.

According to the 2005 ISHLT consensus recommendations, only when there are positive histologic features indicative of AMR, further immunohistochemical testing is required. On the basis of our experience, histology per se is unable to identify C4d-positive patients or patients with AMR because less than half of the positive patients show any histologic features indicative of AMR. The fact that our data on CD68 at immunohistochemistry showed no differences in the presence of a prominent accumulation of intravascular macrophages in the four groups supports the hypothesis that C4d should be performed on a routine basis. Immunohistochemical CD68 detection is not indicative of AMR. C4d-positive patients could not be identified at histology, and no correlation between histology and clinical status could be elicited. At the moment, there are no standardized and internationally recognized criteria for interpretation and reporting of C4d staining. The international community is still involved in guidelines for C4d testing. We have chosen to adopt the grading of Chantranuwat et al. because it represents the most frequent adopted scheme in the literature for paraffin-embedded samples, and it turned out to be an easy and practical tool.

Controversy continues to grow with respect to the significance of C4d positivity and consequently to patient management both in the early and late posttransplantation stages because little data are available, especially with respect to paraffin-embedded tissue (*37*, *38*). Our findings indicate that C4d positivity is associated with a poor outcome. If and how these patients should be treated is another controversial issue, but plasmapheresis, intravenous immunoglobulins, and rituximab could be an initial therapeutic approach. Close surveillance is mandatory to detect early signs of graft dysfunction.

In contrast with previous studies (*39*–*42*) showing a correlation between AMR and onset of CAV and between AsAMR and asymptomatic CAV, we were unable to find any correlation between C4d positivity and development of CAV. However, we detected only DSA, no other type of antibody that has been recognized as potential triggers of AMR. We cannot exclude that lack of correlation could be ascribed to the presence of no-complement fixed antibodies or to the numerosity of the groups, to the recognized low sensitivity of angiography to detecting CAV, or even to the fact that C4d at immunoperoxidase is less sensitive than at immunofluorescence (*43*). The relatively small number of patients evaluated and the fact that circulating DSA were not routinely assessed in the C4d-negative patients are all limitations of this study. Notwithstanding these constraints, the implications of the use of C4d immunostaining in the surveillance of HTx warrant consideration and further studies.

In conclusion, our finding indicate that AMR is a complex and ongoing phenomenon with different phenotypic features. C4d positive predicts worse prognosis, and DSA and graft dysfunction further improve risk stratification. C4d positive and DSA can be used as early mortality predictors in patients without signs of graft dysfunction.

MATERIALS AND METHODS

Patient Population

A total of 107 adult patients (n=85, 79% of these were males) with a median age at the time of transplantation of 55 years (range: 17–73 years) participated in the study. Sixteen (15.0%) of these were transplanted less than a year earlier, whereas 55 (51.4%) had undergone transplantation 1 to 5 years earlier. The remaining 36 (33.6%) had undergone transplantation more than 5 years earlier. After receiving information about the aims and procedures of this study, the patients signed informed consent forms.

C4d staining on EMBs has been routinely performed in our center as a part of our posttransplant monitoring protocol since 2004. We evaluated 985 consecutive biopsies from 107 transplant patients (median=8 biopsies per patient). EMBs were performed in accordance with the protocol schedule (*44*) and graded for ACR using the 1990 ISHLT biopsy grading scale, as indicated elsewhere (*14*) until 2004 and 2005 ISHLT scale after that date (*45*). After the first year, EMBs were performed at 1-year interval. Hematoxylin and eosin biopsy stains taken before 2004 were regraded using the 2005 ISHLT scale. A rejection score, based on a modification of the ISHLT classification, was assigned as follows: 1R=1, 2R=2, and 3R=3. The following scores were calculated for each patient: the total rejection score calculated taking into consideration the total number of scores registered during the follow-up, and severe total rejection score calculated taking into consideration the scores equal to or above 2R. All the scores were normalized for the total number of biopsies performed in each patient by dividing each score by the total number of EMBs performed during the study period.

In accordance with the ISHLT revised consensus criteria, AMR was diagnosed on the basis of evidence of graft dysfunction, circulating antidonor antibodies (DSA), histologic evidence of acute capillary injury, CD 68 intracapillary positivity, and C4d capillary positivity on EMBs (*46*, *47*). However, National Institute of Health classification of AMR proposed sequence of stages of AMR: latent, subclinical, and clinical (*29*). C4d immunostaining was carried out during routine surveillance controls, performed in accordance with the protocol described earlier. In the presence of C4d positivity, DSA were also determined at the same time.

Our study population was divided into four groups on the basis of the patients' C4d, DSA, and graft function profile:

- C4d positive, DSA negative, without graft dysfunction (AsAMR)= group 1.
- C4d positive, DSA positive, without graft dysfunction (AsAMR)= group 2.
- C4d positive, DSA positive, presence of graft dysfunction (symptomatic AMR)=group 3.
- C4d negative, DSA negative, without graft dysfunction, considered the control group=group 0.

Specimen Handling and Processing

Right ventricular EMB specimens were taken at established intervals using the percutaneous transcatheter method (*48*, *49*) (see **Supplemental Digital Content 7,** http://links.lww.com/TP/A237).

Immunohistochemistry Staining Technique

Histopathologic evaluation and C4d staining were performed on formalin-fixed, paraffin-embedded sections (see **Supplemental Digital Content 8,** http://links.lww.com/TP/A238 and **Supplemental Digital Content 9,** http://links.lww.com/TP/A239).

DSA Assessment

IgG antihuman leukocyte antigens reactivity in the sera, obtained before transplantation and at the time of C4d-positive detection on EMBs, was analyzed using bead-based screening assays, referred to as Luminex methodology (*50*, *51*) (see **Supplemental Digital Content 10,** http://links.lww.com/TP/A240).

Clinical Data and CAV Assessment

The patients' clinical data collected during the follow-up ending in December 2008 were reviewed. Cardiac allograft dysfunction was defined as the finding of left ventricular ejection fraction below 50%, measured by transthoracic echocardiography or by sign and symptoms of heart failure. Ninety-five of the 107 patients studied underwent annual coronary angiography after heart transplantation. CAV was assessed in accordance with the criteria established by Gao et al. (*52*). Immunosuppressive drugs such as cyclosporine and mycophenolate mofetil with or without corticosteroids were used. Only few cases were on everolimus or azathioprine.

Statistical Analysis

Qualitative data were expressed as counts and percentages, whereas quantitative data were expressed as medians and ranges (minimum and maximum), because they were not normally distributed. The comparison between C4d groups was conducted with the Fisher exact test in case of categorical variables and the Kruskal-Wallis test for quantitative variables. The Kaplan-Meier method was applied to estimate the C4d groups' survival functions, and the log-rank test was used to compare survival between groups. The Cox's regression model was used to estimate unadjusted and sex- and age-adjusted hazard ratios with the 95% CI considering the C4d occurrence a time-dependent covariate in the posttransplantation period. All the tests were two tailed, and a P value of 0.05 was considered statistically significant. The statistical analyses were performed using SAS 9.1.3 for Windows (SAS Institute Inc., Cary, NC).

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