Original Research

Pattern and Evolution of C4d Staining of Ischemic Myocardial Injury: Implications for the Interpretation of Post-Transplant Endomyocardial Biopsies

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C4d immunohistochemical staining is a marker of recent classical pathway complement activation that is useful for evaluation of antibody-mediated rejection in transplant biopsies. C4d also stains areas of myocyte necrosis. We describe the pattern and intensity of myocyte, interstitial, and microvascular staining at different stages of ischemic injury/infarction in the non- transplant setting. Thirty autopsies with ischemic injury were reviewed. Nine acute myocardial infarction, 3 contraction band necrosis, 9 subendocardial ischemic, and 9 chronic ischemic injury/scarring cases were stained with polyclonal antibody for C4d. Results: Acute myocardial infarction and subendocardial ischemic injury cases showed strong staining of necrotic myocytes; larger infarcts showed more intense peripheral versus central staining. Subendocardial ischemic injury was easier to quantify versus H&E staining. Necrosis with contraction bands was highlighted in individual myocytes. Two of 9 cases of chronic ischemic injury/scarring showed only rare positive cells. C4d was noted to highlight amyloid in 4 cases. Microvascular staining was noted in only 2 cases, was faint and not associated with injured areas. Autolysis had no effect on staining. C4d is a useful diagnostic tool to highlight necrotic myocytes, especially in the absence of large areas of obvious necrosis. It can be used to differentiate true from artifactual contraction band injury and can be used on autolyzed material. Microvascular staining is not seen around areas of infarction. This finding may help in the interpretation of perioperative ischemic injury versus humoral rejection in heart transplants, wherein microvascular staining in post-implantation biopsies should prompt additional clinical investigations to rule out humoral rejection.
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Key Words: *C4d, immunohistochemical staining technology, heart disease, myocardial infarction (MI)*

INTRODUCTION

Cardiovascular diseases are the leading causes of death worldwide. In particular, coronary heart disease, or ischemic heart disease, caused an estimated 7.2 million deaths in 2004.[1] Ischemic heart disease presents as myocardial infarction (MI), angina pectoris, heart failure, or sudden cardiac death and may be caused by coronary atherosclerosis, emboli, or decreased systemic blood pressure as seen in states of shock.[2]

At autopsy, the gross and microscopic appearance of an MI depends on the duration of survival post-MI. Early recognition of an acute MI can be a challenge to pathologists, especially when the post-MI survival is less than 12 hours. On gross examination, MIs are usually not apparent when less than 12 hours old, although early coagulation necrosis may be seen microscopically between 4 and 12 hours post-MI.[2] Myocardial infarctions that are less than 4 hours old are usually not apparent microscopically on hematoxylin and eosin (H&E)-stained sections.

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One method used to demonstrate areas of infarction as early as 2 to 3 hours post-MI is the staining of fresh heart tissue sections with triphenyl tetrazolium chloride (TTC). The TTC reaction depends on dehydrogenase enzymes within the myocytes. Normal myocardial tissue will stain purple-red, while infarcted tissue that has no dehydrogenase enzyme activity, either due to leakage or exhaustion of stores from within dead cells, will not stain with the pigment.[3,4] The use of TTC in the macroscopic diagnosis of acute MI has been shown to have a sensitivity and specificity of 77.4% and 92.6%, respectively.[4] However, autolysis may lead to the disappearance of dehydrogenases in normal myocardium from 36 to 60 hours post-mortem depending on the tissue temperature, thereby causing a false non-deposition of TTC.[5]

Immunohistochemical staining techniques may also be helpful in establishing a diagnosis of early acute MI by highlighting myocytes with ischemic injury or necrosis. Studies have shown that antibodies against C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1), fibronectin, and the complement split products C5b-9, C9, and C4d will stain areas of myocyte necrosis in infarcts of varying ages.[6-11] Currently, positive staining for C4d in the

microvasculature is used as a marker of antibody-mediated (humoral) rejection in the evaluation of cardiac and kidney transplant biopsies.[12,13] The purpose of our study is to document the pattern and intensity of myocyte and interstitial staining with C4d at different stages of ischemic injury/infarction in the non-transplant setting. We particularly note the pattern of any associated microvascular staining, which affects the differential diagnosis of humoral rejection versus perioperative ischemic injury in the post-transplant setting.

METHODS

Complete autopsies, including clinical data from hospital medical records, of patients with myocardial ischemic injury performed at Robert Wood Johnson University Hospital, New Brunswick, NJ, in 2008 and 2009 were reviewed. Restricted autopsies that did not include examination of thoracic organs were excluded. A total of 30 cases were identified. This study was approved by the institutional review board at Robert Wood Johnson Medical School.

Gross findings of the heart recorded by the prosector at the time of the autopsy were reviewed. H&E stained slides of the myocardium were also reviewed, and representative paraffin-embedded sections from each case were stained with a polyclonal antibody for C4d (DAKO North America, Carpinteria, CA) using a Ventana Benchmark XT automated immunostainer (Ventana Medical Systems, Inc, AZ). Slides were cut at 3-4 microns, deparaffinized and tissue conditioning performed with EDTA buffer (pH 8.0-8.5) for 30 minutes. Antibody detection is performed with the ultraview Universal DAB kit on the ventana immunostainer (Ventana Medical Sytstems, Inc. AZ). Positive control tissue for C4d was a renal allograft with antibody mediated rejection.

RESULTS

Thirty cases including 15 men and 15 women with an age range of 28 to 89 years were evaluated. The race was Caucasian for 19, African-American for 4, Hispanic for 3, and other/unknown for 4 cases. The degree of coronary atherosclerosis was none for 6 (20%), mild for 5 (17%), moderate for 3 (10%), and severe for 16 (53%) cases. Systemic atherosclerosis was present in 14 (47%) of the 30 cases. The cause of death was cardiac-related in 12 (40%) of the 30 cases. See **Table 1**.

The cases were categorized according to the histologic findings as follows: Acute MI (N = 9) defined as areas of coagulative myocyte necrosis involving more than the inner 1/3 of myocardium; Subendocardial ischemic injury (N = 9) defined as area of coagulative myocyte necrosis limited to the inner 1/3 of the ventricular wall, generally small, non-confluent; Contraction band injury (N = 3) defined as foci of myocytes demonstrating hypereosinophilic stripes or bands within the cytoplasm morphologically diagnostic for contraction bands; and Chronic ischemic injury/scarring (N = 9) defined as areas of granulation tissue (with or without residual recognizable necrotic myocytes) to frank areas of well formed scar tissue. The clinicopathologic correlates with C4d staining results are tabulated (**Table 1**). The gross findings of the hearts as described by the prosector correlated with the histologic findings in 8 of the 9 (89%) cases of acute MI. Four of 9 (44%) subendocardial ischemic injury, 1 of 3 (33%) contraction band only injury, and 3 of 9 (33%) chronic ischemic injury cases appeared grossly negative for acute myocardial ischemia.

All nine cases with acute MI showed at least focally strong C4d staining in necrotic myocytes, with larger infarcts showing more intense peripheral versus central staining (**Figure 1 A&B**). All nine cases with subendocardial ischemic injury showed strong, homogeneous staining outlining the focus of ischemic injury, making it easier to quantify versus H&E staining alone. None of the surrounding normal, uninjured myocytes stained for C4d. Two of the 3 cases of contraction band injury showed C4d positivity in individually necrotic myocytes (**Figure 1 C&D**). One case with contraction bands noted on H&E was negative for C4d and was subsequently reclassified as having artifactual contraction bands. Two of the 9 cases of chronic ischemic injury/scarring showed rare C4d-positive cells. One case of chronic ischemic injury/scarring with severe autolysis/decomposition showed no non-specific staining for C4d.

Microvascular staining with C4d, as typically seen in antibody-mediated rejection in heart transplant biopsies,[12] was noted in 2 cases: 1 with subendocardial ischemic injury and 1 with chronic ischemic injury. The staining was patchy and faint and not localized to injured areas. Both of these patients had active infections at the time of death. Unexpectedly, amyloid was strongly positive for C4d in all 4 cases (**Figure 1 E&F**). Two of these cases were initially considered to be scarring and were reclassified after additional histochemical staining with congo red (Ventana Medical Systems, Inc, Tuscon, AZ) and immunostaining with amyloid P protein (Novacastra Laboratories LTD, Newcastle upon Tyne, United Kingdom).

DISCUSSION

In this study, we evaluated the pattern of C4d staining in different stages of myocyte ischemic injury/infarction at autopsy. We found that C4d shows strong positivity in necrotic myocytes in areas of ischemic injury/infarction, with stronger staining at the periphery of larger areas of infarction. C4d also shows positive staining in myocytes with true contraction band injury. Surrounding normal myocytes do not stain with C4d, which allows for easier quantification of the area of injury versus H&E staining. Areas of ischemic injury do not show C4d positivity in the microvasculature. Tissue with autolysis does not stain non-specifically with C4d, as evidenced by our case of chronic ischemic injury/scarring with severe autolysis/decomposition, in which the body was not discovered until 3 to 4 days postmortem. C4d also highlights amyloid deposition.

Table 1. Clinicopathologic and C4d Staining Results.

Case	Age/ Race/ Sex	Cause of Death	Atherosclerosis	Gross Findings	Microscopic Findings	C4d Staining
Acute MI						
1	85 AA F	Arrhythmia status post MI	Severe coronary & systemic	Septal mottling, posterior wall fibrosis	1-3 days old MI	Patchy, transmural & along periphery of infarct
2	54 H M	Acute myeloid leukemia	None	Posterolateral mottling	Acute MI	Patchy, transmural & along periphery of infarct
3	70 C F	Catecholamine-induced toxic myocarditis	Mild coronary	Unremarkable	Toxic myocarditis	Scattered cells, transmural
4	86 C M	Sepsis	Moderate coronary	Posteroseptal fibrosis, adjacent mottling	Acute extension of old MI	Patchy subendocardial
5	67 H F	Ventricular rupture/hemoperi-cardium	Severe coronary & systemic	Laterobasal ventricular rupture	Acute MI with rupture	Patchy, subendocardial & along periphery of infarct
6	71 AA F	Arrhythmia, cor pulmonale	Severe coronary & systemic	Hemorrhagic septum	3-4 days old MI, amyloid	Patchy transmural & amyloid
7	89 C F	MI	Severe coronary & systemic	Red-brown lateral wall	1-2 days old MI	Patchy subendocardial
8	72 C M	MI, cardiogenic shock	Severe coronary	Red-brown & focally tan posterolateral wall & septum	4 week old MI, amyloid	Patchy transmural, along periphery of infarct & amyloid
9	81 C M	Ventricular rupture/hemoperi-cardium	Severe coronary & systemic	Left ventricular rupture	1 week old MI	Patchy, transmural & along periphery of infarct
Subendo-cardial Ischemia						
10	68 C F	Sepsis	Severe coronary	Posterior wall fibrosis	Subendo-cardial ischemia	Patchy subendocardial
11	71 C M	Massive gastrointestinal hemorrhage	Severe coronary & systemic	Anterior wall fibrosis	Subendo-cardial ischemia	Subendocardial
12	51 O F	Hemoperitoneum	None	Unremarkable	Subendo-cardial ischemia	Subendocardial
13	34 O M	Hemoperitoneum	None	Subendo-cardial mottling	Subendo-cardial necrosis	Subendocardial
14	75 C M	Sepsis	Mild coronary & moderate systemic	Unremarkable	Subendo-cardial ischemia & amyloid	Subendocardial & amyloid
15	65 C F	Sepsis	Severe coronary	Posterior wall fibrosis	Subendo-cardial ischemia	Subendocardial

(**Table 1.** Clinicopathologic and C4d Staining Results. Continued …)

Case	Age/ Race/ Sex	Cause of Death	Atherosclerosis	Gross Findings	Microscopic Findings	C4d Staining
Acute MI						
16	29 O M	Cerebrovascular accident, hypertrophic cardiomyopathy	None	Focal fibrosis	Subendocardial ischemia	Subendocardial
17	65 C M	Atrial fibrillation, acute cardiac failure	Mild coronary	Unremarkable	Subendocardial ischemia	Subendocardial & microvasculature
18	53 AA M	Metastatic colon carcinoma	Mild coronary	Unremarkable	Subendocardial ischemia	Subendocardial
Contraction Bands						
19	73 C F	Respiratory failure	Moderate coronary & systemic	Pale posterolateral wall	Contraction band injury	Negative
20	54 C F	Sepsis	Mild coronary	Unremarkable	Contraction band injury	Subendocardial
21	28 C M	Toxic myocarditis	None	Subendocardial mottling	Contraction band injury	Scattered cells with contraction band injury
Chronic Ischemic Injury						
22	71 H F	Metastatic ovarian carcinoma	Severe coronary & systemic	Anteroseptal fibrosis	Scarring	Negative
23	46 C M	Congestive heart failure	Severe coronary & systemic	Anterolateral fibrosis	Remote MI	Negative
24	70 C F	Sudden cardiac death, long QT syndrome	Severe coronary & systemic	Anterior & posterior wall fibrosis	Fibrosis	Microvasculature
25	67 C F	Retroperitoneal hematoma, hypotensive shock	Moderate coronary & systemic	Unremarkable	Scar	Negative
26	61 C F	Massive gastrointestinal hemorrhage	No coronary & moderate systemic	Posteroseptal, posteroinferior & papillary fibrosis	Remote MI	Negative
27	52 C F	Sepsis	Severe coronary	Unremarkable	Chronic ischemia	Negative
28	49 AA M	Hemoperitoneum	Severe coronary	Anterior wall fibrosis	Chronic ischemia	Rare cells
29	86 C M	Sepsis	Severe coronary & systemic	Septal fibrosis	Remote MI, amyloid	Rare cells & amyloid
30	81 O M	Sudden unexpected death in epilepsy	Severe coronary & systemic	Unremarkable	Patchy fibrosis, decomposition	Negative

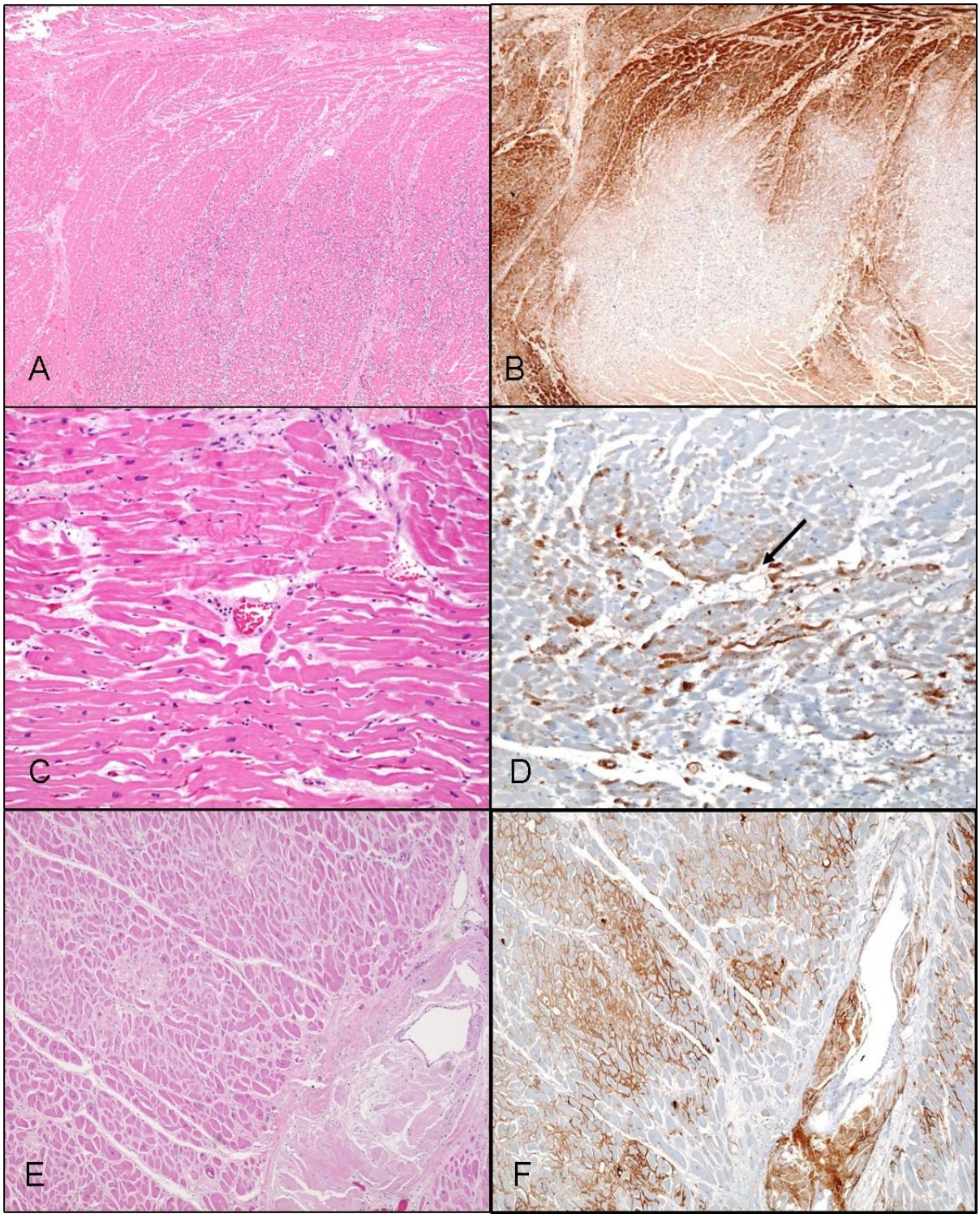

Figure 1.
A. Low power (40X) H+E stained section of acute myocardial infarction.
B. C4d immunohistochemical stain of same infarct demonstrating strong staining of the peripheral myocytes without staining of necrotic myocytes within the center of the large are of necrosis. (C4d immunohistochemical stain 40x)
C. H+E stained section (100x) demonstrating necrotic myocytes with intracytoplasmic hyperosinophilic stripes or bands diagnostic for contraction band necrosis.
D. C4d immunohistochmeical stain of same case demonstrating patchy but strong staining of the contraction band necrosis. (C4d immunohistchemical stain 100x).
E. Low power(40x) H+E stained section of myocardium demonstrating vascular and interstitial accumulation of amyloid material.
F. C4d immunohistochemical stain of same case demonstrating staining of the amyloid material. (C4d immunohistochemical stain 40x).

C4d is an inactive split product of C3 convertase, and deposition in tissues indicates recent classical pathway complement activation. Deposition of C4d in the capillaries of post-transplant endomyocardial biopsies has been shown to correlate with the presence of serum anti-donor antibodies with a sensitivity of 84% and a specificity of 89%, aiding in the diagnosis of antibody-mediated/humoral rejection.[12] Studies suggest that in MIs, CRP activates the classical complement pathway leading to C4d deposition in the myocardium.[6,7,14] This is followed by ICAM-1 expression and neutrophil infiltration, indicating that CRP enhances inflammation, and perhaps infarct size, by activating complement.[7,15]

A recent study by Jenkins, et al evaluated the utility of C4d, C9, and troponin T immunostaining to detect the presence and extent of acute MIs at autopsy.[11] They reported 13 samples with cellular injury, consisting of combinations of contraction bands, hemorrhage, coagulation necrosis, and myocyte hypereosinophilia without neutrophil infiltration, which showed positivity for C4d in injured cells with negativity in adjacent normal myocytes. Twelve samples with only contraction bands suggestive of early ischemic injury were all negative for C4d. In our study, we had 3 cases with contraction band injury only, of which 2 showed positive C4d staining in injured cells. Contraction bands may be seen not only in the ischemia-reperfusion setting, but also in cocaine and catecholamine-induced toxic myocarditis and as an artifact commonly seen in endomyocardial biopsies.[16] Therefore, we interpreted the case with negative C4d staining as artifactual contraction bands not due to myocyte ischemic injury. One of the 3 contraction band only cases was in fact due to toxic myocarditis (**Table 1**). Our other case of toxic myocarditis showed scattered aggregates of C4d-positive myocytes transmurally and was categorized as an acute MI.

Both our study and the study by Jenkins, et al observed that the area of injury of well-developed acute MIs highlighted by C4d was sometimes larger than what was perceived on H&E staining and that C4d may help in the diagnosis of reinfarction or acute extension of an older MI.[11] In the latter study, all of their 14 cases of remote infarctions with scarswere negative for C4d.[11] In our study, 2 of the 9 chronic ischemic injury/scarring cases showed rare positive cells with the remainder of the cases being nonreactive.

None of the 3 previously mentioned studies that utilized C4d immunohistochemistry described the presence or absence of C4d staining in the microvasculature of the myocardium.[6,7,11] As described above, C4d positivity in the capillaries of post-transplant endomyocardial biopsies indicates recent classical pathway complement activation and correlates with the presence of serum alloantibodies.[12] In our study, we observed a lack of C4d microvascular staining in areas of myocyte injury. Two cases showed patchy, faint capillary staining which was not associated with injured areas. Both of these patients had active infections at the time of death. Therefore, we conclude that the microvascular staining in these cases is most likely the result of complement activation by the infectious process.

Interestingly, we noted that C4d highlights amyloid deposition. This phenomenon has been previously reported in the neuropathology literature. Studies have shown that cerebral amyloid deposits, cerebral amyloid angiopathy, and neurofibrillary tangles that are positive for amyloid P by immunohistochemistry are also positive for both C4d and C3d, indicating that amyloid P is associated with classical pathway complement activation.[17,18]

The major limitation of this study is that cases were identified retrospectively. Therefore, the gross findings, including degree of coronary and systemic atherosclerosis and description of the myocardium, and the areas that were sampled for microscopic evaluation are dependant on the prosector at the time of autopsy. Also, the exact interval between cardiac injury and time of death was not recorded for all cases. Therefore, we cannot demonstrate how early C4d will stain injured cells post-MI. Although we did include 1 case with severe autolysis/decomposition, the majority of the autopsies were performed 24 to 48 hours postmortem after immediate refrigeration. Therefore, the effect of autolysis on the specificity of the immunohistochemical stain could not be fully evaluated.

In summary, C4d is a useful diagnostic tool to highlight myocyte ischemic injury especially in the absence of obvious areas of necrosis on H&E staining. It is very useful in quantifying the degree of injury, differentiating true from artifactual contraction band injury, and identifying acute extension of an old MI or reinfarction. Importantly, microvascular staining does not occur in areas of infarction/ischemia. This may help in the interpretation of humoral rejection versus perioperative ischemic injury in post-transplant endomyocardial biopsies, wherein microvascular staining should prompt additional clinical investigations to rule out rejection.

CONFLICT OF INTEREST
None.

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