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## Endothelial Dysfunction and Cytomegalovirus Replication in Pediatric Heart Transplantation

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**Background**—Cardiac allograft vasculopathy is the major limiting factor to the long-term success of pediatric heart transplantation. Cytomegalovirus (CMV) has been shown to be a significant risk factor for the development of cardiac allograft vasculopathy. Recent work has demonstrated CMV DNA in leukocytes in the absence of direct allograft infection, suggesting that vascular changes may not be limited to the allograft.

**Method and Results**—Systemic arterial endothelial function was assessed with high-resolution ultrasound to determine brachial artery flow-mediated dilation in 50 pediatric heart transplant recipients (8 to 17 years of age; 27 male). Patients were separated into 2 groups according to CMV status: those without evidence of CMV replication after transplantation (n=38; 19 male) and patients with evidence of viremia after transplantation (n=12; 8 male). No patient had detectable viremia at the time of study. Flow-mediated dilation was significantly impaired in patients with evidence of CMV replication after transplantation ( $6.64 \pm 1.12\%$ , mean  $\pm$  SE) compared with those without ( $9.48 \pm 0.56\%$ ;  $P=0.02$ ). This difference remained after adjustment for age, time since transplantation, and medication. Pretransplantation recipient and donor CMV status and traditional CMV risk were not associated with flow-mediated dilation.

**Conclusions**—CMV replication after cardiac transplantation is associated with chronic endothelial dysfunction in the systemic circulation in children. The implication for both systemic and coronary vascular health requires prospective evaluation. (*Circulation*. 2008;117:2657-2661.)

**Key Words:** endothelium ■ pediatrics ■ transplantation ■ virus

Although short- and medium-term results are improving, long-term survival for pediatric transplant recipients is limited by cardiac allograft vasculopathy, an accelerated form of obliterative coronary disease. Human cytomegalovirus (CMV) is associated with increased risk of posttransplantation coronary vasculopathy.<sup>1,2</sup> We have shown CMV serology to be an important factor in allograft vasculopathy of pediatric recipients.<sup>3</sup> Coronary endothelial dysfunction is associated with CMV infection<sup>4</sup> and the later development of transplant vasculopathy.<sup>5</sup> Recent work has shown that low-level systemic CMV infection is common after transplantation,<sup>6</sup> although infection of the heart itself is rare.<sup>7</sup> It is therefore possible that the coronary vascular disease attributed to CMV may be driven by the consequences of low-grade systemic infection rather than infection of the transplant organ, which may have broader vascular consequences. However, the effect of posttransplantation CMV on the systemic vasculature is unknown. We tested the hypothesis that CMV replication could affect systemic endothelial function after pediatric cardiac transplantation.

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#### Methods

##### Study Population and Design

All children >8 years of age who had previously had a heart transplant returning to Great Ormond Street Hospital for their annual review between October 2004 and August 2006 were invited to take part in the study. Exclusion criteria were coexisting vascular disease, patient or parent refusal, active intercurrent systemic illness, and clinical or echocardiographic evidence of heart failure (left ventricular fractional shortening <30%). Fifty-two patients were invited, 2 of whom were excluded because of lack of parental consent. The remaining 50 patients (age, 8 to 17 years; 27 male) were enrolled and were separated into 2 groups based on CMV status: group 1 (n=38; 19 male), those without evidence of CMV replication after transplantation (negative polymerase chain reaction [PCR] and no change in CMV IgG after transplantation), and group 2 (n=12; 8 male), patients with evidence of posttransplantation viremia (positive CMV PCR and/or seroconversion from negative to positive CMV IgG). No patient from either group was viremic at the time of assessment. All patients were free of angiographic evidence of cardiac allograft vasculopathy and were assessed for conventional vascular risk

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**Table 1. Baseline Characteristics of Study Participants**

Characteristics	No CMV Replication	CMV Replication	<i>P</i>
Patients, n	38	12	...
Age, y	14.5±0.4	13.1±0.6	0.071
Male sex, n (%)	19 (50)	8 (67)	0.322
Heart rate, median (IQR), bpm	91 (79–104)	89 (84–97)	0.751
Systolic blood pressure, mm Hg	113±2	110±3	0.534
Diastolic blood pressure, mm Hg	71±1	65±3	0.065
Cholesterol, mmol/L	3.12±0.11	3.09±0.21	0.887
Triglycerides, mmol/L	0.88±0.06	1.01±0.14	0.291
HDL, mmol/L	1.02±0.05	1.03±0.07	0.962
LDL, mmol/L	1.73±0.11	1.59±0.19	0.551
Glucose, median (IQR), mmol/L	5.10 (4.80–5.78)	5.20 (4.40–6.00)	0.596
hsCRP, median (IQR), mg/L	0.86 (0.25–3.55)	0.45 (0.11–0.70)	0.108
Creatinine, μmol/L	67.7±2.8	70.6±8.0	0.664

IQR indicates interquartile range; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and hsCRP, high-sensitivity C-reactive protein. All blood tests are fasting values. Values are expressed as mean±SEM or frequency (percentage) unless otherwise specified.

factors (hypertension, smoking, diabetes, lipid profile) and transplantation and medication history.

### Conventional and Transplantation-Related Risk Factors

There were no smokers or diabetics among the cohort. There were no significant differences between the groups with respect to age, sex, heart rate, blood pressure, or blood glucose or lipid levels (Table 1). Renal function, measured by serum creatinine and estimated glomerular filtration rate (data not shown), was similar in both groups.

Transplantation-related variables and drug use were comparable between the groups (Table 2). A double-therapy immunosuppressant regimen of calcineurin inhibitor and a purine synthesis inhibitor was used except when the latter was not tolerated, in which case a single-agent therapy was used. No patients were receiving steroids at the time of the scan. Eleven patients (7 in group 1, 4 in group 2) were donor positive, recipient negative for CMV IgG at transplantation. Of these, 6 (3 in group 1, 3 in group 2) received CMV prophylaxis (valganciclovir 450 mg OD) for 3 months after transplantation.

### Peripheral Endothelial Function Measurement Methods

Endothelial dysfunction was studied as previously described.<sup>8</sup> Briefly, the subjects rested supine for 10 minutes, having abstained

from caffeine and fatty food for 4 hours before the scan. The right brachial artery was then imaged with high-resolution ultrasound (Prosound SSD-5500, ALOKA, Tokyo, Japan). Forearm ischemia was induced by inflating a blood pressure cuff to 200 mm Hg for 5 minutes; reactive hyperemia followed cuff deflation. Changes in brachial artery diameter were measured offline with an automated edge detection system (Brachial Tools, Medical Imaging Applications, Coralville, Iowa) and calculated as a percentage change from baseline diameter. Blood flow was measured continuously with a pulsed-wave Doppler signal. Maximal increase in blood flow within 15 seconds of cuff release was expressed as a percentage change from baseline flow (±SE). Endothelium-independent vasodilator response to 25 μg sublingual glyceryl trinitrate (GTN) was then measured and expressed as a percentage change in diameter from baseline. Blood pressure was initially taken after resting for 5 minutes and after flow-mediated dilation (FMD) and GTN studies with an OMRON M5-I sphygmomanometer (OMRON, Kyoto, Japan). All studies were performed in a temperature-controlled vascular laboratory by a single trained operator who was blinded to all clinical history of the patients, including CMV variables.

### CMV Measurements

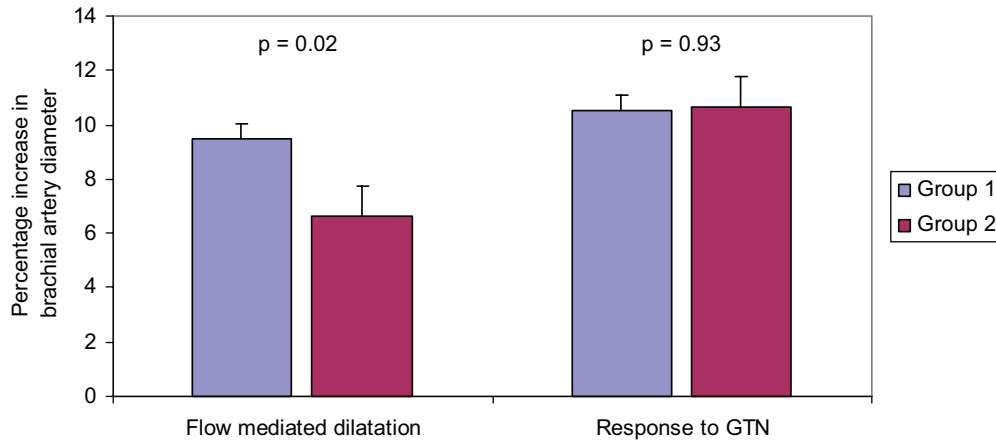
Blood for CMV analysis was taken before transplantation, at monthly clinic visits after transplantation in the first year, and annually thereafter. DNA was extracted from 200 μL EDTA whole blood with QI Amp Blood Mini Kits (catalog No. 80204, QIAGEN Corp, Valencia, Calif) according to the manufacturer's instructions. CMV DNA was quantified in whole blood by a method developed by J. Garson and R. Tedder at University College Hospital, London (oral communication, 2003) that was standardized against samples distributed by the European Quality Control for Molecular Diagnosis program.

The master mix for the reaction consisted of 10 μL Taqman universal master mix (catalog No. 4304437, ABI Biosystems, Forest City, Calif) for tests run on the ABI 7000 and 7300 machines or 10 μL Taqman fast master mix (ABI catalog No. 4352042) for tests run on the ABI 7500 machine: 1 μL (10 pm/μL) CMV forward primer, GCA TGC GCG AGT GTC AAG AC; 1 μL (10 pm/μL) reverse primer, GTT ACT TTG AG(CT) GCC ATC TGT TCC T; 2 μL (10 pm/μL) probe, FAM-TGC GCC GTA TGC TGC TCG ACA-TAMRA; 1 μL nuclease free water; and 10 μL DNA template. Cycling conditions were as follows: ABI 7000 and ABI 7300: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds, and a final step at 60°C for 1 minute; and ABI 7500 fast machine: 1 cycle at 5°C for 20 seconds followed by 45 cycles at 95°C for 3 seconds and a final step at 60°C for 30 seconds. Viral loads were obtained by reference to 10 CMV (strain AD169 culture in human embryo lung fibroblasts) standards that were included in each run, ranging from 100 to 2 million copies per 1 mL extract. Assays were considered valid if the correlation coefficient for the standards was >0.99 and the gradient was between -3.3 and -3.6. CMV IgG was measured by a Vidas machine (bioMérieux, Marcy l'Etoile, France).

**Table 2. Transplant-Related Variables**

Characteristics	No CMV Replication	CMV Replication	<i>P</i>
Age at transplant, median (IQR), y	11 (6–15)	9 (7–13)	0.555
Donor age, y	19.3±2.0	21.7±4.3	0.579
Ischemic time, min	196±11	156±18	0.081
Episodes of acute rejection, median (IQR), n	0 (0–1)	0 (0–1)	0.644
Time since transplant, median (IQR), y	3.63 (1.40–7.97)	2.68 (1.58–5.07)	0.674
Statin use, n (%)	34 (90)	9 (75)	0.337
Double immunosuppression, n (%)	34 (90)	10 (83)	0.621

IQR indicates interquartile range. Values are expressed as mean±SEM or frequency (percentage) unless otherwise specified.



**Figure.** FMD and GTN response (mean with SE) in group 1 (n=38; no posttransplantation CMV replication) and group 2 (n=12; positive CMV PCR or seroconversion after transplantation).

**Statistical Analysis**

Data were analyzed with SPSS 13.0 for Windows (SPSS Inc, Chicago, Ill). Two-group comparisons of means were performed with the unpaired Student *t* test for normally distributed data (age, cholesterol, triglycerides, creatinine, blood pressure, donor age, ischemic time, FMD, and dilatation to GTN) and the Mann–Whitney *U* test for nonnormally distributed data (age at transplantation, time since transplantation, episodes of acute rejection, heart rate, serum glucose, and high-sensitivity C-reactive protein) after testing for normality with the Kolmogorov-Smirnov test. Multiple-group comparisons were conducted with a 1-way ANOVA (CMV risk stratification). Comparisons of proportions were calculated with Fisher’s exact test (sex, medications). Linear regression with multiple predictors was performed to explore the relationship between CMV status and FMD with adjustment for age, time since transplantation, and medication.

The study was approved by the Great Ormond Street Hospital Ethics Committee. Informed consent was obtained from all patients’ parents, and consent/assent was taken (as appropriate) from the patients themselves.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Vascular Function**

No patients were CMV PCR positive at the time of the brachial scan. The mean time from last positive PCR to scan was 23 months, and only 2 patients had a positive PCR within the 6 months before the scan. Brachial artery FMD was significantly reduced in patients with previous active CMV viremia after transplantation compared with patients without evidence of replication (6.64±1.12% versus 9.48±0.56%; *P*=0.02; the Figure). This difference remained significant when adjusted for age, time since transplantation, and medication (*P*=0.02). In contrast, response to GTN was comparable between the 2 groups, indicating that the difference in FMD was not due to differences in smooth muscle function. Baseline arterial diameter (2.96±0.079 versus 3.23±0.12 mm; *P*=0.094) and flow (reactive hyperemia, 489±41% versus 437±128%; *P*=0.618) also were similar in groups 1 and 2.

The patients also were analyzed according to their donor CMV status, recipient pretransplantation status, and traditional CMV risk stratification<sup>9</sup> (Table 3). None of these 3

variables was predictive of FMD. There was no correlation between maximum CMV PCR detected or duration of PCR positivity and FMD.

**Discussion**

The present study is the first to link CMV and systemic vascular dysfunction after heart transplantation. It demonstrates that children with evidence of CMV replication after heart transplantation (as shown by positive PCR or seroconversion) have significantly worse systemic endothelial function. Although impaired endothelial dysfunction has been described in children with symptoms of acute viral illnesses,<sup>10</sup> our patients had neither signs of infection nor evidence of CMV DNA in their blood at the time of vascular study, suggesting a more chronic association between CMV and vascular function. The relationship between active CMV replication and reduced FMD remained after adjustment for multiple potential confounding variables. The young age of the cohort minimizes the impact of many of the potential confounders for systemic vascular disease such as smoking, hypertension, and diabetes. In addition, there was no relationship to known risk factors such as inflammation (high-sensitivity C-reactive protein), lipids, or blood pressure. This abnormality in endothelial function may have important implications for the later systemic vascular health of young

**Table 3. FMD Grouped by Pretransplantation CMV Variables**

Criterion	Value	n	FMD, %	<i>P</i>
Donor CMV status	Positive	19	9.03±0.89	0.911
	Negative	29	8.90±0.67	
Recipient CMV status	Positive	15	8.82±0.72	0.840
	Negative	33	9.06±0.70	
Traditional risk stratification	High risk	11	9.46±1.40	0.928
	Intermediate risk	16	9.00±0.69	
	Low risk	20	8.95±0.85	

High risk indicates donor CMV positive and recipient CMV negative; intermediate risk, recipient CMV positive; and low-risk, donor and recipient CMV negative. FMD values are mean±SEM. The donor CMV status was not available in 2 cases. The recipient’s pretransplantation status was not available in 2 cases.

transplant recipients and may reflect changes in the coronary circulation of relevance to the pathogenesis of cardiac allograft vasculopathy.

Although systemic vascular health clearly is important for children after transplantation, it is coronary vasculopathy that limits their long-term outcome.<sup>11,12</sup> The pathogenesis of this disease is complex, multifactorial, and currently incompletely understood. However, studies have highlighted the relationship between CMV and allograft vasculopathy in adults<sup>1,2,13</sup> and, more recently, in children.<sup>3</sup> One of the hallmarks of allograft vasculopathy is endothelial dysfunction.<sup>5</sup> Although the mechanisms by which CMV may cause vasculopathy are unknown, it is likely that dysfunction of the endothelium is important. CMV has been shown to infect endothelial cells<sup>14</sup> and is related to coronary vasomotor dysfunction in heart transplantation patients.<sup>4</sup> We have now extended these findings to the systemic vasculature.

In previous adult studies, CMV-negative recipients of CMV-positive hearts have shown an increased cardiovascular risk.<sup>15,16</sup> In our study, no relationship was seen between systemic endothelial function and donor or recipient CMV status, the traditional markers of risk of CMV disease. CMV prophylaxis has reduced the risk of coronary disease in this group.<sup>17</sup> However, in most patients, no prophylaxis is given, and CMV infection involving leukocytes remains common.<sup>6</sup> Evidence is accumulating that graft vasculopathy may be driven by the consequences of low-grade systemic infection and reduced by its suppression.<sup>17</sup> Pretransplantation CMV seropositivity (and consequent lack of aggressive prophylaxis) appears to be associated with coronary lumen loss.<sup>18</sup> This is consistent with our previous work on CMV and graft vasculopathy in children<sup>3</sup> and with our current findings in which viral DNA was documented in patients CMV IgG positive before transplantation. That subclinical CMV infection may have a systemic endothelial effect is further illustrated by our finding that 11 of the 12 children with posttransplantation CMV replication did not go on to develop overt CMV disease yet showed reduced systemic endothelial function.

A recent study by Tu et al<sup>13</sup> has investigated the immune response to subclinical CMV infection in recipients who were CMV IgG positive at transplantation. This study showed that patients with CMV-specific CD4 t cells in the first month after transplantation showed less coronary artery lumen loss than those without these cells. These “early responders” also were found to have low or undetectable levels of CMV by PCR (mean, <40 copies per  $1 \times 10^5$  polymorphonuclear cells compared with >250 copies per  $1 \times 10^5$  polymorphonuclear cells in those without the early response). Although our study was not designed to explore t-cell responses and the study populations are different in terms of CMV status at transplantation, it is probable that there is overlap of our group without detectable virus and the early responder group of Tu et al. We hypothesize that similar immune mechanisms may be working in our study, protecting against systemic vascular injury. A follow-up project investigating immune responses and systemic arterial function is required to explore this theory in detail. However, both our results and the work by Tu et al demonstrate that subclinical CMV infection is associated

with later vascular abnormalities and that those without evidence of viral replication have a more favorable vascular outcome.

The precise mechanism by which prior CMV replication causes ongoing endothelial dysfunction is unclear. It is possible that low-level CMV viremia (undetectable by current assays) or persistent infection of leukocytes or endothelial cells affects the vasculature directly. Alternatively, either of these mechanisms may impair endothelial function indirectly through chronic immune activation. It also is possible that our observations are a lasting consequence of a prior endothelial insult sustained at a time of higher viral load.

Quantification of endothelial function with ultrasound assessment of brachial artery FMD is a well-validated, noninvasive method. Recently, the first large, prospective study has shown FMD to be predictive of future cardiovascular events in adults.<sup>19</sup> The prognostic value in younger patients remains unproven because the cardiovascular end points typically present decades later. FMD also is predictive of coronary endothelial function in adults,<sup>20</sup> raising the possibility that depressed FMD in these children reflects impaired coronary endothelial function relevant to the progression of cardiac allograft vasculopathy. Certainly, a noninvasive marker of transplant coronary vasculopathy would be diagnostically attractive, particularly in the pediatric age group. Furthermore, because FMD is a dynamic measurement, it may be useful as a therapeutic marker in clinical trials assessing the response to CMV infections in the transplantation population.

All children in the present study had angiographically normal coronary arteries, although a more sensitive assessment of the coronary vasculature such as intravascular ultrasound would have been useful. Although this technique is limited by the size of coronary arteries in young children, it is possible in older children and adolescents. The monitoring of CMV viral load was limited to outpatient visits, and it is possible that some patients could have experienced low-level CMV replication that was not detected; we accounted for this possibility in part by checking for seroconversion in those patients who were CMV negative at transplantation.

## Conclusion

We have shown that CMV replication after cardiac transplantation is associated with chronic endothelial dysfunction in the systemic circulation in children. The implication for both systemic and coronary vascular health requires prospective evaluation.

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## Disclosures

None.

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### CLINICAL PERSPECTIVE

Cardiac allograft vasculopathy, the major limiting factor to the long-term success of pediatric heart transplantation, is associated with the development of endothelial dysfunction in the donor coronary arteries. Cytomegalovirus (CMV) has been shown to be a significant risk factor for the development of cardiac allograft vasculopathy. Recent work has demonstrated CMV DNA in leukocytes in the absence of direct allograft infection, suggesting that vascular changes may not be limited to the allograft. The present project used flow-mediated dilation in the brachial artery to illustrate decreased native endothelial function in children who had shown CMV replication after heart transplantation. Importantly, none of the patients enrolled in the present study were CMV DNA polymerase chain reaction positive at the time of their scan, and only 1 developed clinical CMV disease. In addition, it was interesting to note that neither the pretransplantation CMV status of donor or recipient nor CMV mismatch was associated with changes in brachial endothelial function. The present work demonstrates that the effects of posttransplantation CMV are not limited to the allograft and that traditional risk factors for CMV disease may not be adequate to identify those patients who are most likely to suffer CMV-mediated complications. It also promotes the idea that subclinical CMV replication may be an important influence on later vascular health, even after virus is no longer detectable in the blood. Further studies are required to prove a link between systemic endothelial dysfunction and the later development of cardiac allograft vasculopathy in these patients.