Cytomegalovirus Exposure and Cardiovascular Disease in Kidney Transplant Recipients

Cécile Courivaud,1,2,3 Jamal Bamoulid,1,2,3 Jean-Marc Chalopin,1,2,3,4 Emilie Gaiffe,4 Pierre Tiberghien,1,2,4,5 Philippe Saas,1,2,4,5 and Didier Ducloux1,2,3,4

1UMR1098, INSERM, 2IFR133, Université de Franche-Comté, 3Department of Nephrology, Dialysis, Renal Transplantation and 4CIC-BT 506, CHU Besançon, and 5Plateforme de Biomonitoring, CIC-BT 506, EFS Bourgogne Franche-Comté, Besançon, France

(See the editorial commentary by Shimamura on pages 1487–90.)

Some data suggest that cytomegalovirus (CMV) may be involved in atherogenesis. However, there are few data suggesting that CMV may contribute to posttransplantation atherosclerosis. We studied a cohort of 570 consecutive renal transplant recipients. The impact of CMV on atherosclerotic events was analyzed with respect to other known main cardiovascular risk factors. The mean follow-up duration (±SD) was 87 ± 31 months. A total of 357 patients were considered to be CMV exposed, and 213 were considered to be CMV naive. Cox regression analysis revealed that CMV exposure (hazard ratio [HR], 1.80 [95% confidence interval {CI}, 1.06–3.05]; P = .030) was an independent risk factor for atherosclerotic events. A total of 213 patients remained CMV negative during follow-up, 225 CMV-positive patients had no replication after transplantation, and 132 CMV-positive patients experienced CMV replication after transplantation. Atherosclerotic event rates were 8.5%, 13.3%, and 18.2%, respectively (P = .034). Cox regression analysis revealed that patients with posttransplantation CMV replication had an increased risk of atherosclerotic events (HR, 2.06 [95% CI, 1.03–4.15]; P = .042) and death (HR, 1.76 [95% CI, 1.08–2.89]; P = .024). There was also a trend toward an increased risk of atherosclerotic events in CMV-positive patients without posttransplantation replication (HR, 1.62 [95% CI, .91–3.05]; P = .098). Both pretransplantation CMV exposure and posttransplantation CMV replication contribute to the increased risk of cardiovascular disease in transplant recipients.

Keywords. cytomegalovirus; kidney transplantation; cardiovascular disease.

It is now widely accepted that the initiation and progression of atherosclerotic lesions involves a chronic inflammatory response. However, the continuous source of the inflammation is still unclear. Some evidence suggests that infectious agents may contribute to inflammation in the plaques. Among them, cytomegalovirus (CMV) has been linked for decades to cardiovascular disease (CVD) [1–3] and heart transplant vasculopathy [4, 5]. Several mechanisms have been evoked to explain this association. CMV has been shown to directly infect human vessels [6, 7], leading to smooth muscle cell proliferation and migration, uptake of oxidized low-density lipoprotein, release of cytokines and chemokines, and increased procoagulant activity by endothelial cells [8–11]. Alternatively, CMV may induce vascular lesions without direct invasion. Viral antigens can trigger an immune response that cross-reacts with self-peptides expressed on vascular wall [8]. Finally, CMV is a driver of age-associated immune changes, which lead to a reduction of naïve T cells, an expansion of terminally differentiated T cells, and an increase in proinflammatory cytokines [12].

Both CMV disease [13] and atherosclerotic events [14] are very prevalent in kidney transplant recipients. However, there are few data suggesting that CMV exposure may contribute to so-called posttransplantation accelerated atherosclerosis. A previous study reported...
that CMV disease is associated with cardiac complications in kidney transplant recipients [15], but this study involved patients who were not receiving CMV prophylaxis, analyzed both atherosclerotic and nonatherosclerotic cardiac events, and did not consider pretransplantation CMV exposure. As a consequence, the results are probably no longer appropriate to the current practice and concerns. In this article, we report results of a study conducted to assess whether pretransplantation CMV exposure and/or posttransplantation CMV replication may be risk factors for posttransplantation atherosclerosis.

METHODS

Study Design and Populations
We analyzed a cohort of 570 consecutive renal transplant recipients who underwent transplantation at University Hospital of Besançon between January 1993 and December 2008. The ethics committee of Franche Comté approved the study.

All the patients received quadruple sequential immunosuppression. Induction was performed with either antithymocyte globulin (ATG; 351 patients [62%]), consisting of ATG-Fresenius (Fresenius) for 225 patients (64%); 9 mg/kg on day 0 and 3 mg/kg/d on days 1–4) or Thymoglobulin (Genzyme) for 126 patients (36%; 2 mg/kg on day 2 and 1 mg/kg/d on days 1–4), or with monoclonal anti-CD25 antibody (Simulect; Novartis) for 219 (40%; 2 mg/kg on day 0 and 20 mg on day 4). The same maintenance immunosuppressive treatments were used, including cyclosporine (January 1993–July 2001) or tacrolimus (August 2001–December 2008), azathioprine (January 1993–October 2000) or mycophenolate mofetil (November 2000–December 2008), and steroids.

All patients except CMV-seronegative recipients of a CMV-seronegative donor kidney (D−/R−) received CMV prophylaxis with valacyclovir in the first 3 months following transplantation. The antiviral prophylaxis dose was adapted to renal function. All patients received Pneumocystis antimicrobial prophylaxis with trimethoprim-sulfamethoxazole. Characteristics of the study population are described in Tables 1 and 2.

CMV Infection and Disease
Serologic analysis for CMV was performed by enzyme-linked immunosorbent assay before transplantation. The donor CMV serology was assessed through medical records. Polymerase chain reaction (PCR) analysis for CMV was performed weekly until month 3 after transplantation, monthly from 3–6 months after transplantation, and each year during follow-up. Patients were considered to have CMV infection if PCR results were positive. CMV disease was defined as the need of treatment by a patient with viral replication. CMV exposure was defined as a positive results of pretransplantation CMV serologic analysis and/or posttransplantation CMV infection or disease.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exposed (n = 357)</th>
<th>Naive (n = 213)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>48 ± 13</td>
<td>45 ± 15</td>
<td>.039</td>
</tr>
<tr>
<td>Male sex</td>
<td>229 (64)</td>
<td>148 (69)</td>
<td>.200</td>
</tr>
<tr>
<td>First transplant</td>
<td>307 (86)</td>
<td>188 (88)</td>
<td>.641</td>
</tr>
<tr>
<td>Preformed HLA antibodies</td>
<td>64 (17.9)</td>
<td>23 (10.8)</td>
<td>.023</td>
</tr>
<tr>
<td>ATG use</td>
<td>227 (64)</td>
<td>124 (58)</td>
<td>.123</td>
</tr>
<tr>
<td>Past history of CVD</td>
<td>48 (13.4)</td>
<td>20 (9.3)</td>
<td>.148</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>38 (10.6)</td>
<td>18 (8.5)</td>
<td>.395</td>
</tr>
<tr>
<td>Hypertension</td>
<td>295 (83)</td>
<td>171 (80)</td>
<td>.734</td>
</tr>
<tr>
<td>Tobacco consumption</td>
<td>92 (25.7)</td>
<td>50 (23.5)</td>
<td>.543</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>117 (32.7)</td>
<td>60 (28)</td>
<td>.250</td>
</tr>
<tr>
<td>Body mass index, mean ± SD</td>
<td>24.1 ± 4.4</td>
<td>23.4 ± 4.4</td>
<td>.108</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>86 (24.1)</td>
<td>52 (24.4)</td>
<td>.930</td>
</tr>
</tbody>
</table>

Data are no. (%) of patients or mean value ± SD.

Table 1. Characteristics of Patients, by Cytomegalovirus Exposure Status

Potential Confounding Factors
Age, sex, weight, size, hemodialysis duration before transplantation, pretransplantation CVD, diabetes mellitus, hypercholesterolemia, hypertension, body mass index, smoking status, panel reactive antibody, rank of transplantation (first vs iterative), donor type, and immunosuppressive treatment (type of induction, cyclosporine vs tacrolimus, azathioprine vs mycophenolate mofetil) were assessed.

Atherosclerotic Events
Coronary Heart Disease
Myocardial infarction was documented by serial 12-lead electrocardiogram evidence or Q-wave infarction and elevations in relevant myocardial enzymes; by coronary revascularization, including coronary artery bypass surgery or percutaneous transluminal coronary angioplasty; or by a typical history of angina with abnormal coronarography findings.

Stroke/Cerebrovascular Disease
Both nonhemorrhagic and hemorrhagic strokes were confirmed by neurologic examination findings consistent with new onset focal neurologic deficits, with or without computed tomography or magnetic resonance imaging evidence of cerebral infarction; or by a history of symptomatic extracranial artery stenosis resulting in carotid endarterectomy.

Abdominal Aortic or Lower Extremity Arterial Disease
Abdominal aortic or lower extremity arterial disease were confirmed on the basis of a history of abdominal aortic repair, lower extremity revascularization via bypass surgery or angioplasty, lower extremity amputation, or new onset of intermittent claudication confirmed by Doppler or arteriography findings.
Two physicians independent of the study were responsible for diagnostic ascertainment. This analysis was performed without knowledge of baseline characteristics.

**Immunee Exhaustion**

Absolute numbers of circulating B and T cells, CD4+ T cells, and CD8+ T cells were determined as previously described [16] in 75 consecutive patients from the main cohort. The mean time since transplantation (±SD) was 62 ± 21 months. Naive CD4+ T cells were also assessed as CD45RA+, CD62L+, CD45RO−, and CD3+ cells, using the following antibodies: FITC-conjugated CD45RA (clone HI100), phycoerythrin-CD62L (Dreg56; BD Biosciences, Le Pont de Claix, France), ECD-CD45RO (UCHL1), PC7-CD3 (13B8.2), and allophyco-cyanin-CD3 (UCHT1; Beckman Coulter). Exhausted T cells were assessed as CD57+CD28− T cells [17], using the following antibodies: FTIC-conjugated CD57 (Beckman Coulter) and CD28 perCP/Cy5.5 (BD Biosciences Pharmingen). Naive T cells were defined as CD45RA+CD28+ T cells.

**Inflammation**

C-reactive protein (CRP) levels were measured by nephelometry (Kit Beckman) at the end of follow-up for surviving patients. The last available CRP measurement was used for patients who died or lost their graft during the study period.

**Statistical Analysis**

We separated patients with any contact with CMV (ie, transplant recipients who had CMV infection or disease before transplantation [R+] and transplant recipients who developed CMV infection or disease after transplantation [R−]) from those who had never been exposed to CMV (ie, D−/R− patients and transplant recipients who did not develop CMV infection or disease after transplantation and had CMV-positive donors [D+/R−]). Arithmetic means and SDs were calculated where indicated.

Using log-rank tests on Kaplan-Meier nonparametric estimates of the survival without cancer distribution, we selected variables with a P value of ≤.20. The selected variables were included into a Cox proportional hazards model, and a backward stepwise selection process was performed, this time at a classical α level of 0.05. Because sex and age were potential confounding variables, they were also entered into the Cox model, no matter the significance of their relationships with death. Tobacco consumption was accounted for by using currently smoking and nonsmoking as variables.

Results are expressed as hazard ratios (HRs) and 95% confidence intervals (CIs), with P values calculated to test the null hypothesis that HRs are equal to 1. Therefore, a P value of <.05 indicates a HR significantly different from 1: a HR of >1 indicates an increased risk of death, and a HR of <1 indicates a decreased risk of death. Assumptions of Cox models (ie, log linearity and proportionality of risk in time) were met in this analysis.

Counts of T-lymphocyte subsets and levels of CRP were compared between CMV-exposed and CMV-naive patients, using the Mann-Whitney U test; corresponding values for patients who remained CMV negative during follow-up, CMV-positive patients without replication after transplantation, and those with CMV replication after transplantation were compared using the Kruskal-Wallis test.

**RESULTS**

**Study Population**

Characteristics of patients, stratified by CMV exposure status, are depicted in Table 1. The mean age (±SD) was 47 ± 14
years. The patients were followed for a mean duration (±SD) of 87 ± 31 months.

CMV Exposure
A total of 252 patients (44%) were CMV seronegative at transplantation. Of these, 122 (48%) received a CMV-seropositive kidney. CMV infection was not detected in D−/R− patients, but 39 D+/R− patients (32.8%) experienced CMV infection or disease. Among 318 patients with positive results of CMV serologic analysis at transplantation, 178 (56%) received a CMV-positive kidney. There was a similar rate of CMV disease or infection in D+/R+ patients (53 cases [29.7%]) and D−/R+ patients (39 cases [27.8%]). Overall, 357 patients were considered CMV exposed (318 were R+, and 39 were R−), and 213 were considered CMV naive (ie, R−). Compared with CMV-naive patients, CMV-exposed patients were older (mean [±SD], 48 ± 13 vs 45 ± 15 years; P = .039) and more likely to have preformed anti-HLA antibodies (17.9% vs 10.8%; P = .023). Therefore, these 2 variables were forced in the Cox model. CMV infection or disease developed after transplantation in 132 patients. Compared with patients who were CMV negative throughout the study, these patients were older (P = .017) and were more likely to have received ATG (71% vs 59%; P = .006).

Atherosclerotic Events
Seventy-two atherosclerotic events (12.6%) occurred during follow-up. This corresponds to 18 atherosclerotic events per 1000 patients/year.

CMV Exposure
The cumulative incidence of atherosclerotic events was higher in CMV-exposed patients (15.2% vs 8.5%; P = .021; Figure 1A). Concerning CMV-naive patients, there was no difference between D−/R− patients and D+/R− patients without replication (9% vs 7.4%). In univariate analysis, age (P < .001), sex (P = .092), a past history of CVD (P < .001), pretransplantation diabetes (P = .003), hypercholesterolemia (P = .010), and CMV exposure (P = .021) were associated with atherosclerotic events. Cox regression analysis revealed that age (HR, 1.02 per 1-year increase [95% CI, 1.00–1.04]; P = .047), a past history of CVD (HR, 1.93 [95% CI, 1.04–3.60]; P = .039), and CMV exposure (HR, 1.80 [95% CI, 1.06–3.05]; P = .030) were independent risk factors for atherosclerotic events (Table 2). Overall model fit (by the Hosmer-Lemeshow test) indicated a χ² statistic of 31 (P < .0001). The area under the curve was 0.66 (95% CI, 0.62–0.70) without CMV exposure and 0.70 (95% CI, 0.66–0.73) with addition of CMV exposure (P = .081). The mean difference (±SD) was 3.2% ± 1.9%. The results were quite similar when D−/R− patients were used as a control group (HR, 1.58 [95% CI, 1.02–2.59]; P = .045; Figure 1B).

CMV Replication
To clarify whether CMV exposure or CMV replication after transplantation was the risk factor for atherosclerotic events, we then considered 3 groups of patients: patients who were CMV negative during follow-up (n = 213), CMV-positive patients without replication after transplantation (n = 225), and CMV-positive patients who had CMV replication after transplantation (n = 132). Atherosclerotic events rates were 8.5%, 13.3%, and 18.2%, respectively, in the 3 groups (P = .034; Figure 1C). In univariate analysis, both CMV-positive patients without replication (HR, 1.76 [95% CI, .97–3.18]; P = .063) and those with CMV replication (HR, 2.37 [95% CI, 1.20–

Figure 1. A, Atherosclerotic event–free survival in transplant recipients who did not develop CMV infection or disease after transplantation and had CMV-negative donors (D−/R−) and in other donor/recipient pairs (ie, who developed CMV infection or disease before transplantation and had CMV-positive donors [D+/R+], those who developed CMV infection or disease before transplantation and had CMV-negative donors [D−/R+], and those who did not develop CMV infection or disease after transplantation and had CMV-positive donors [D+/R−]). B, Atherosclerotic event–free survival in patients who were CMV negative during follow-up (n = 213), CMV-positive patients without replication after transplantation (n = 225), and CMV-positive patients with CMV replication after transplantation (n = 132).
4.70; \( P = .013 \) had an increased risk of atherosclerotic events. Because CMV replication was closely related to ATG use, we analyzed the potential interaction between CMV replication and ATG on atherosclerotic event occurrence. Adjustment on the basis of ATG use did not modify the relationship between CMV replication and atherosclerotic events (HR, 2.28 [95% CI, 1.18–4.79]; \( P = .019 \)). Therefore, to avoid bias inherent to ATG indication, this variable was not forced in the model. Cox regression analysis revealed that patients with posttransplantation CMV replication (HR, 2.06 [95% CI, 1.03–4.15]; \( P = .042 \)) had an increased risk of atherosclerotic events. There was also a trend toward an increased risk of atherosclerotic events in CMV-positive patients without posttransplantation replication (HR, 1.62 [95% CI, 0.91–3.05]; \( P = .098 \); Table 2). Overall model fit (by the Hosmer-Lemeshow test) indicated a \( \chi^2 \) statistic of 32 (\( P < .0001 \)).

**Death**

Seventy-four patients (13%) died during follow-up. Of these, 36 (49%) died of CVD, 26 (36%) died of cancer, 8 (10%) died of infections, and 4 (5%) died of miscellaneous causes. We analyzed whether CMV exposure and/or CMV replication may influence posttransplantation mortality. CMV exposure only marginally increased the risk of death (HR, 1.64 [95% CI, 0.98–2.74]; \( P = .060 \)). By contrast, patients with posttransplantation CMV replication (HR, 2.19 [95% CI, 1.22–3.92]; \( P = .001 \)) had an increased risk of death. CMV positivity without posttransplantation replication was not predictive of death (HR, 1.24 [95% CI, 0.75–2.05]; \( P = .395 \)). Cox regression analysis revealed that posttransplantation CMV replication (HR, 1.76 [95% CI, 1.08–2.89]; \( P = .024 \)) and older age (HR, 1.07 [95% CI, 1.04–1.09]; \( P < .001 \)) were predictive of death. We finally analyzed a combined end point of atherosclerotic events or death (122 events). CMV replication (HR, 1.54 [95% CI, 1.04–2.31]; \( P = .038 \)), but not CMV exposure, was a risk factor for atherosclerotic events or death.

**Immune Exhaustion, Inflammation, and CMV Exposure**

The frequency of CD8\(^+\)CD57\(^+\)CD28\(^-\) T cells among all CD8\(^+\) T cells was greater among CMV-exposed patients (\( n = 41; 18.1\% \) [range, 2.7%–70.1%]) than among CMV-naive patients (\( n = 34; 6.1\% \) [range, 0.1%–46.5%]; \( P < .001 \)). A similar difference was observed for CD8\(^+\)CD57\(^+\)CD28\(^-\) T cells (3.9% [range, 0.1%–36.4%] and 0.3% [range, 0%–39%], respectively; \( P < .001 \)). We then considered 3 groups of patients: patients who were CMV negative during follow-up (\( n = 34 \)), CMV-positive patients without replication after transplantation (\( n = 22 \)), and those with CMV replication after transplantation (\( n = 19 \)). The frequency of CD8\(^+\)CD57\(^+\)CD28\(^-\) T cells among all CD8\(^+\) T cells gradually increased among the 3 groups, with values of 6.1% (range, 0.1%–46.5%), 13.8% (range, 2.7%–60.4%), and 23.7% (range, 3.6%–70.5%), respectively (\( P < .001 \);

**DISCUSSION**

Our study is the first to demonstrate that CMV exposure is predictive of major cardiovascular events in kidney transplant recipients. Among CMV-exposed patients, those with posttransplantation replication seem to have a further increase in risk. Our results also suggest that CMV is associated with immune exhaustion and inflammation. The combined data suggest that infection with CMV is more likely to have an indirect effect on atherosclerosis progression than a direct effect.

Previous studies reported conflicting results about a possible correlation between CMV antibodies and CVD. Small sample size of studies and incomplete confounder adjustments has hampered their significance. Furthermore, most studies have considered transplant arteriosclerosis and restenosis and not native atherosclerosis. However, a large prospective study failed to show an association between CMV antibody presence and atherosclerotic events [18]. Subsequent studies have demonstrated that the amount of CMV antibodies is a crucial determinant. High, but not low, anti-CMV antibody levels are associated with coronary artery disease [1, 19, 20]. Nevertheless, more recently, a recent large cohort study (they National Health and Nutrition Examination Survey) revealed an increased risk of cardiovascular mortality in CMV-seropositive patients.
subjects with high CRP levels [21]. We did not analyze the amount of anti-CMV antibodies as a potential covariate. We cannot exclude that, as in the general population, the intensity of the anti-CMV response may influence the risk of CVD in transplant recipients.

We observed an increased rate of atherosclerotic events in patients with posttransplantation CMV replication as compared to those without replication. CMV can directly invade cardiovascular tissues [6, 7, 19]. As a consequence, CMV reactivation may accelerate the atherogenic process by increasing the detrimental effects of tissue invasion, such as smooth muscle cell proliferation and migration [8–10]. Alternatively, CMV reactivation may stimulate immune responses and inflammation and indirectly contribute to atherosclerosis progression. Indeed, we observed a higher rate of CD8+CD57+CD28− T cells in patients with posttransplantation CMV replication. It has been shown that most of these memory T cells are part of large clonal expansions that are specific for persistent viruses, mainly CMV [20]. Moreover, CRP levels at the end of follow-up were higher in this subgroup of patients. This suggests that repeated immune challenges drive a proinflammatory response, which promotes and/or aggravates atherosclerosis.

A large body of literature suggests that cellular immune response against CMV, and not CMV infection per se, is implicated in the proatherogenic effects of CMV. Hsue et al [21] reported that CMV-specific T-cell responses were independently associated with intima-media thickness in patients with human immunodeficiency virus (HIV) infection. The same group recently reported that the progression of atherosclerosis in HIV-infected patients is associated with a high frequency of CMV-specific CD4+CX3CR1+ T cells [22]. Furthermore, these cells may induce endothelial cells to secrete CX3CL1, which itself drives progressive infiltration of the arterial wall by proinflammatory cells and promotes atherosclerosis. Similarly, Kaplan et al [23] showed that T-cell activation predicts carotid artery stiffness in HIV-infected women. Betjes et al [24, 25] also reported a strong association between CMV seropositivity, terminally differentiated CD4+ T cells, and atherosclerotic disease in patients with end-stage renal disease. Together, these studies demonstrate that cellular immune responses directed against CMV contribute to atherosclerosis. We speculate that adding CMV seropositivity and/or the frequency of anti-CMV T cells may help to better predict cardiovascular events in transplant recipients.

We found posttransplantation CMV replication to be predictive of death. No death was directly related to CMV disease. CMV replication was mainly associated with cardiovascular death (data not shown). Such a relationship between CMV and death has been described in the general population [26]. The longitudinal Swedish OCTO/NONA studies involving elderly patients also determined the existence of an immune risk profile (IRP) predicting mortality [27]. The IRP is characterized by a CD4/CD8 ratio of <1 and the accumulation of late-stage differentiated CD8+ T cells, many of which are specific for CMV antigens. Consistent with this, CMV infection is found to be part of the IRP [12]. A direct relationship between high levels of anti-CMV antibody and death rate has been recently reported in elderly patients [28]. Conversely, a prospective study reported the absence of IRP in centenarians over at least the previous 6 years and probably throughout life [29].

If CMV replication is a risk factor for posttransplantation atherosclerotic events, one can speculate that primary prevention, and not preemptive treatment, of CMV infection would reduce the rate of cardiovascular events in renal transplant recipients. The literature is poor regarding this intriguing question. Spinner et al [30] recently analyzed in a small cohort the long-term effects of these 2 strategies on different posttransplantation outcomes. Cardiovascular events were numerically more frequent in patients having received preemptive therapy (23.4% vs 18.7%). Moreover, a meta-analysis suggests that only universal prophylaxis, and not a preemptive strategy, reduces the death rate in solid-organ transplant recipients [31]. Nevertheless, a long-term randomized study is lacking to confirm this hypothesis.

To conclude, both pretransplantation CMV exposure and posttransplantation CMV replication contribute to the increased risk of CVD in transplant recipients. Further long-term studies are required to establish whether preventing posttransplantation CMV replication could reduce cardiovascular events.

Note

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Potential conflicts of interest. All authors: No reported conflicts.

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