Monitoring Human *Cytomegalovirus* (HCMV) in HCMV-Seropositive Orthotopic Liver-transplant Recipients by Means of Quantitative Real-time Polymerase Chain Reaction

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Objective: **Human *Cytomegalovirus* can be reactivated after orthotopic liver transplantation in patients who are seropositive for *cytomegalovirus*. Whether those *cytomegalovirus*-seropositive patients require immediate posttransplant (anti)-*cytomegalovirus* prophylactic therapy or preemptive treatment as opposed to deferred treatment remains controversial. The aims of our study were to evaluate the relevance of *cytomegalovirus* monitoring with quantitative real-time polymerase chain reaction in whole blood and to analyze the factors that determine the treatment of the first episode of *cytomegalovirus* infection with intravenous ganciclovir in seropositive liver-transplant patients.**

Patients and Methods: **Forty-two *cytomegalovirus*-seropositive liver-transplant patients were assessed for *cytomegalovirus* DNAemia every 2 weeks until posttransplant day 90 and every 3 to 4 weeks until day 180. Biochemical and hematologic parameters were also prospectively monitored.**

Results: ***Cytomegalovirus*** **DNAemia was detected at least once in 27 patients (64%). Treatment was initiated in 12 patients (group 1) but not in 15 others (group 2). Median HCMV viral loads of the first positive and the highest DNAemia were statistically higher in group 1 than in group 2 (*P* = 0.01). Univariate analysis of DNAemia showed that alkaline phosphatase levels were significantly higher in group 1 than in group 2 (*P* = .0011) and that hemoglobin levels were significantly lower in group 1 than in group 2 (*P* = .0443). The results of multivariate analysis showed that the only factor that predicted the treatment of the first episode of HCMV DNAemia was a level of alkaline phosphatase greater than 150 IU/L [odds ratio, 20; range, 1.97-203.32; *P* = .01].**

Conclusions: **A combination of criteria, including viral-load kinetics, clinical factors, alkaline phosphatase levels (in particular), and the patient's immune condition, is required to efficiently monitor patients who are seropositive for *cytomegalovirus* after orthotopic liver transplantation.**

Key words: *Human cytomegalovirus, Liver transplantation, Quantitative real-time PCR, HCMV DNAemia, Alkaline phosphatase*

The potent immunosuppressive therapy received by orthotopic liver transplant (OLT) recipients potentiates the development of opportunistic infections such as that caused by human *cytomegalovirus* (HCMV), which may be manifested as HCMV syndrome or symptomatic end-stage organ dysfunction. In particular, symptomatic infection may lead to the development of acute or chronic graft rejection and increased mortality [1-3].

The incidence of HCMV disease is higher in seronegative recipients (R-) who have received an organ from a seropositive donor (D+) (26%-70%) than in seropositive recipients (4.5%-10%) [2, 4-9]. To prevent that disorder, transplant recipients may benefit from prophylaxis with anti-HCMV drugs (valacyclovir, ganciclovir, or, more recently, valganciclovir) during the first 3 or 4 months after transplantation [10-12]. In both R+ and R- recipients, prophylactic therapy seems more effective than deferred therapy in decreasing the incidence of HCMV disease and delaying its onset [13], although some clinical studies have suggested that late-onset HCMV develops despite prophylaxis [2]. Moreover, there is some debate as to whether prophylaxis should be given universally or should be targeted to high-risk groups (ie, D+/R- patients) [9]. If prophylaxis is not given,

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HCMV viremia can be monitored and the patients treated when it becomes positive, i.e., pre-emptive therapy [14-16] or, conversely, when no HCMV monitoring is performed the patients are treated when clinical symptoms occur, i.e., deferred therapy [17].

It seems reasonable to assume that in cases of low-risk HCMV infection (ie, in seropositive recipients), no prophylaxis is required but either preemptive treatment or symptom-triggered treatment should be administered [18], and ganciclovir and valganciclovir have been shown to be effective in such patients [14-16].

In patients who receive preemptive treatment, regular clinical, biologic, and virologic follow-ups are necessary during the first months after transplantation to detect early HCMV infection (ie, HCMV DNAemia) and to initiate treatment, even in the absence of clinical manifestations. Although the patients who receive preemptive treatment are monitored on a regular basis, it is often not clear when to initiate treatment, and the early detection of HCMV infection with sensitive and predictive methods is necessary. Accurate diagnosis, which involves the use of good positive and negative predictive values, depends on the type of organ transplantation as well as the kind of virologic test used.

Numerous virologic techniques, such as pp65 antigenemia [19] and, more recently, quantitative PCR [20] or quantitative real-time PCR on plasma [21], are available to monitor OLT patients. Quantitative real-time PCR on plasma has also been shown to indicate the development of HCMV disease. Moreover, recent studies [20] have demonstrated the need for a the rigorous assessment of OLT patients, especially by means of a survey of viral-load kinetics between the date of the first DNA detection and week 4 after transplantation. We have previously described monitoring performed by means of quantitative real-time PCR from DNA that was automatically extracted from whole blood (WB) with a MagNA Pure instrument (Roche Molecular Biochemicals, Mannheim, Germany) [22]. We, like other investigators, have demonstrated that there is a good correlation between the HCMV pp65 antigenemia assay and the quantitative real-time PCR test in leukocytes and in whole blood. We have demonstrated that a viral load of 4 $\log_{10}$/mL (10,000 copies/mL) corresponds to 50 positive pp65 cells and that this value could be the threshold needed to initiate anti-HCMV preemptive treatment in HCMV-seropositive renal transplant recipients [23, 24].

In D+/R- OLT patients, anti-HCMV treatment with intravenously administered (IV) ganciclovir is initiated as soon as the results of testing for the markers of HCMV infection are positive, even in the absence of clinical manifestations. To the best of our knowledge, no published results report the monitoring of HCMV infection in R+ patients by means of quantitative real-time PCR or the assessment of the cutoff point above which anti-HCMV therapy should be initiated.

Therefore, the aims of our study were to describe the clinical, biologic, and virologic HCMV monitoring of R+ OLT patients and to evaluate the relevance of HCMV monitoring with quantitative real-time PCR. In addition, the viral loads determined at the onset of HCMV DNAemia in patients who subsequently were or were not treated with IV ganciclovir were compared. We also analyzed the factors that determined the initiation of treatment of the first episode of HCMV infection. For this purpose, we compared the 2 groups' clinical, biologic, and virologic data obtained during the study.

Patients and Methods

During 2002 and 2003, 70 patients underwent liver transplantation at our institution. Of those, 42 were HCMV-seropositive recipients at the time of transplantation, and they did not receive anti-HCMV prophylaxis. The study was approved by the local ethics committee, and all patients gave their written informed consent. During the follow-up of those individuals, HCMV infection was monitored by the regular sampling of whole blood. HCMV DNAemia was assessed every 2 weeks until posttransplant day 90 and thereafter at 3-week to 4-week intervals until day 180. At the same time, we assessed the patients' alanine (ALT), aspartate aminotransferase (AST), gamma-glutamyl-transpeptidase (γGT), alkaline phosphatase (AP), serum creatinine, and hemoglobin levels and their platelet, white blood cell (WBC), and polymorphonuclear-cell (PMN) counts.

The reactivation of HCMV infection was defined as at least 1 episode of DNAemia during the first 6 months after transplantation. Symptomatic HCMV disease was defined according to established criteria [25], which included a fever higher than 38°C for 48 hours in the absence of bacterial or fungal infection or liver rejection, a neutrophil count that progressively decreased over 3 days, thrombocytopenia defined as $< 100 \times 10^9$/L, and/or the involvement of an organ.

Established criteria for anti-HCMV treatment

When HCMV DNAemia was diagnosed, the decision

to treat the patient with IV ganciclovir (10 mg/kg/d for 7-21 days according to renal function) was based on the following criteria: an HCMV viral load greater than 10,000 copies/mL (4 $\log_{10}$) with or without fever, the identification of HCMV DNAemia in 2 consecutive blood samples, leucopenia (a white blood cell count < 3000/mm^3), neutropenia (a polymorphonuclear cell count < 1500/mm^3), or an increase in the ALT level. In the event of a positive test result for HCMV DNAemia, baseline immunosuppression was not modified.

Immunosuppression
Most patients received induction therapy with anti-CD25 monoclonal antibodies (basiliximab or daclizumab). All patients received a steroid pulse (IV methylprednisolone 500 mg) before transplantation and were then treated with the following therapy: prednisolone 20 to 60 mg/d from days 1 to 14, 15 to 30 mg/d from days 15 to 30, 15 to 20 mg/d from days 31 to 60, 10 to 15 mg/d from days 61 to 90, and 10 mg/d after day 91. On day 1 after transplantation, all patients received a calcineurin inhibitor agent that was in most cases tacrolimus with or without mycophenolate mofetil 2 g/d.

When acute rejection was suspected, the patients underwent a liver biopsy, the results of which were read by the same pathologist. If acute rejection was confirmed by means of the Banff 97 criteria, the patient was treated with IV methylprednisolone 10 mg/kg/d for 3 consecutive days, and tacrolimus was adjusted in order to achieve trough levels of ~15 ng/mL. In cases of steroid-resistant acute rejection, in addition to intravenous steroids, the patients received either polyclonal antithymocyte antibodies for 6 consecutive days or monoclonal anti-CD3 antibodies 10 mg/d for 10 consecutive days.

Biologic parameters
The following values for hepatic or hematologic abnormalities and renal dysfunction were assessed in each patient at weekly intervals up to posttransplant day 30, every other week up to day 90, and then every 3 weeks up to day 180: liver enzymes (ALT, AST, γGT, AP, and total bilirubin); hemoglobin; WBC, PMN, and platelet counts; serum creatinine level; and calculated creatinine clearance (Cockcroft and Gault formula).

Virologic methods
Serial samples were collected into potassium EDTA tubes for quantitative real-time PCR, which was performed with DNA extracted from whole blood as previously described [22]. The detection limit of the method was 500 copies/mL (2.69 $\log_{10}$/mL).

Serologic markers
HCMV serology was assessed in the donor as well as in the recipient on the day of transplantation and 6 months after surgery. IgG and IgM were detected with the ETI-CYTOK-G Plus and ETI-CYTO-M reverse assays (DIA Sorin, Antony, France) according to the manufacturer's instructions.

Statistical analyses
All results are expressed as the mean ± standard deviation or as median (range) values. Qualitative variables were compared with the Yates corrected chi-squared test. Quantitative variables were compared with the Mann-Whitney *U* test. *P* values < .05 were considered significant.

Results

Seventy patients underwent OLT transplantation at our institution during 2003 and 2004. Of those patients, 42 (17 women and 25 men; median age, 54 years) were HCMV seropositive at the time of transplantation. Twenty-one patients received a seropositive transplant, and 21 received a seronegative transplant. The median follow-up, which included 380 whole-blood collections, was 220 days (range, 16-790 days). Viral monitoring was performed by the previously described quantitative real-time PCR method. HCMV infection was detected in 27 patients (64%; 17 men and 10 women; median age, 56 years; age range, 35-68 years; 91 of 380 samples). According to the established criteria, 12 of those patients were treated (group 1). Of those, all but 1 patient received IV ganciclovir for either 15 days (7 patients) or for 18 to 21 days (4 patients). Valacyclovir was given to 1 patient for 124 days. Fifteen patients did not meet the specified criteria and were not treated (group 2). Eleven of the infected patients received a seronegative transplant, and the other 16 patients (9 in group 1 and 7 in group 2; *P* = ns) received a seropositive transplant. Immunosuppressive treatment was not modified in any of the infected patients. The rate of acute rejection after positive CMV DNAemia was 33% in group 1 and 13.3% in group 2 (*P* = ns)

Of the treated patients, 10 had hepatic dysfunction (6 patients had an increase in both the ALT and the γGT value, 3 patients had an increase in the γGT value, and 1 patient had an increase in the ALT value). The remaining 2 patients were treated because they had a viral load > 4 $\log_{10}$ copies/mL. One of those patients had associated leucopenia, and the other had a fever. In the group of 15 untreated patients, 2 had an increase in the ALT value and 1 patient had both cytolysis and leucopenia.

Results of biologic evaluations

We compared the liver enzyme levels and hematologic parameters of the 2 groups at the time of their first episode of HCMV viremia. Univariate analysis showed that the AP levels were significantly higher in group 1 than in group 2 (571 [range, 149-994] and 142 [range, 87.3-197] IU/L, respectively; $P = .0011$) and that the hemoglobin levels were significantly lower in group 1 than in group 2 (11 [range, 10-12] and 12 [range, 11-13) g/dL , respectively; $P = .0443$; Table 1). Multivariate analysis showed that AP levels > 150 IU/mL increased the risk of needing treatment (odds ratio, 20; range, 1.97-203.32; $P = .01$).

Virologic results

The median time of virologic follow-up was 178.5 days (range, 36-790 days) in HCMV-infected patients, 180 days (range, 48-790 days) in group 1, and 191 days (range, 36-515 days) in group 2. One hundred forty samples were tested in group 1, and 132 were tested in group 2. DNAemia was detected in 59 samples in group 1 and in 32 samples in group 2. HCMV was detected in a single sample from 7 patients and in up to 20 samples from 1 patient. The distribution of positive and negative samples for each patient in each group is shown in Table 2.

The median time between the time of transplantation and the first HCMV-positive DNAemia test was 34 days (range, 11-109 days); it was 36.5 days (range, 22-109 days) in group 1 and 33 days (range, 11-100 days) in group 2 (P = ns). The median time between the time of transplantation and the highest DNAemia value was 40 days (range, 18-109 days); it was 39.5 days (range, 30-109 days) in group 1 and 42 days (range, 18-100 days) in group 2 (P = ns; Table 1).

The median HCMV viral load, regardless of the treatment group and the time at which DNAemia was identified, was 962 copies/mL (range, 3-626,000 copies/mL); ie, 3 $\log_{10}$ copies/mL (range, 0.48-5.80 $\log_{10}$ copies/mL). The median HCMV viral load was 1045 copies/mL (range, 3-626,000 copies/mL); ie, 3.07 $\log_{10}$ copies/mL (range, 0.48-5.80 $\log_{10}$ copies/mL) in group 1 and 692 copies/mL (range, 14-41,665 copies/mL); ie, 2.68 $\log_{10}$ copies/mL (range, 1.15-4.61 $\log_{10}$ copies/mL) in group 2 (P = .05). The median HCMV viral load of the first positive DNAemia was 2820 copies/mL (range, 50-626,000 copies/mL); ie, 3.45 $\log_{10}$ copies/mL (range, 1.69-5.80 $\log_{10}$ copies/mL) in group 1 and 330 copies/mL (range, 14-8700 copies/mL); ie, 2.52 $\log_{10}$ copies/mL (range, 1.15-3.94 $\log_{10}$ copies/mL) in group 2 (P = .01). The median HCMV viral load of the highest DNAemia value was 8817 copies/mL (range, 50-626,000 copies/mL); ie, 3.95 $\log_{10}$ copies/mL (range, 1.69-5.80 $\log_{10}$ copies/mL) in group 1 and 500 copies/mL (range, 14-41,665 copies/mL); ie, 2.70 $\log_{10}$ copies/mL (range, 1.15-4.62 $\log_{10}$ copies/mL) in group 2 (P = .01) (Figure 1, A-C).

A good response was shown in the decrease and eradication of HCMV in blood from those in group 1. Ten of the 12 treated patients in that group demonstrated clearance of HCMV. However, the absence of a significant decrease in HCMV DNAemia was

Table 1. Univariate analysis of factors associated with the first episode of HCMV DNAemia

Variables	Group 1 (n = 12)	Group 2 (n = 15)	*P* Value
Median time to first HCMV DNAemia episode (day)	36.5 ± 22.4	33 ± 27.9	ns
Median time to highest HCMVDNAemia value (day)	39.5 ± 21.3	42 ± 25.6	ns
Median viral load ($\log_{10}$ copies/mL) of the first DNAemia episode	3.45 ± 2	2.52 ± 1.43	.01
Median viral load ($\log_{10}$ copies/mL) at the highest DNAemia value	3.95 ± 2.14	2.70 ± 1.86	.01
Tacrolimus trough levels (ng/mL)	9.9 ± 4.6	11.8 ± 3.7	ns
Steroid (mg/kg/d)	0.43 ± 0.26	0.32 ± 0.18	
γGT (IU/L)	856 ± 1060	150 ± 154	ns
Alkaline phosphatase (IU/L)	571 ± 629	142 ± 98	.001
Total bilirubin (μmol/L)	68.5 ± 115	26 ± 42	ns
Hemoglobin (g/dL)	11 ± 1	12 ± 2	.04
WBCC (per mm^3)	6255 ± 2138	6347 ± 3125	ns
Platelets (per mm^3)	177,000 ± 72,000	169,000 ± 69,000	ns

HCMV, human cytomegalovirus; γGT, gamma glutamyl transpeptidase; WBCC, white blood cell count

Table 2. Distribution of positive and negative test results in the study subjects

Group 1 (*n* = 12)		Group 2 (*n* = 15)	
Number of Positive Samples	Number of Positive Samples	Number of Positive Samples	Number of Positive Samples
2	5	1	5
2	4	2	3
2	9	7	5
20	5	3	15
8	11	3	6
3	4	1	6
4	4	3	2
2	6	1	10
11	9	1	5
2	8	1	5
2	11	3	11
1	5	1	7
		3	6
		1	4
		1	10
59	**81**	**32**	**100**

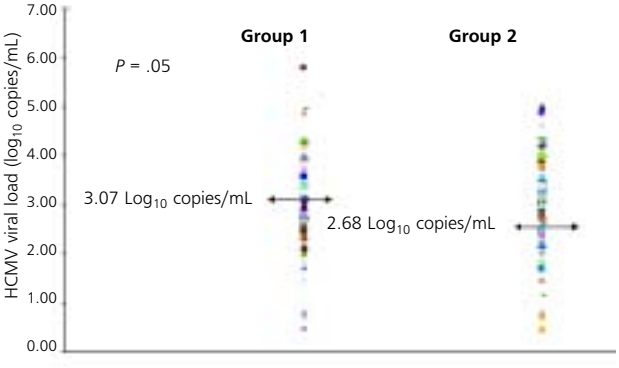

Figure 1A. HCMV, Human *Cyomegalovirus* viral load in treated vs. untreated patients.

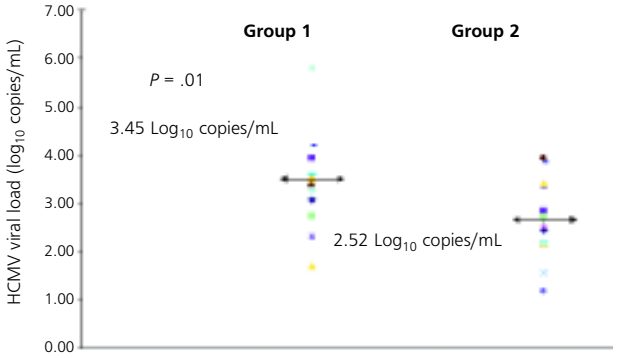

Figure 1B. HCMV, Human *Cyomegalovirus* viral load at the moment of the first positive DNAemia test in treated vs untreated patients.

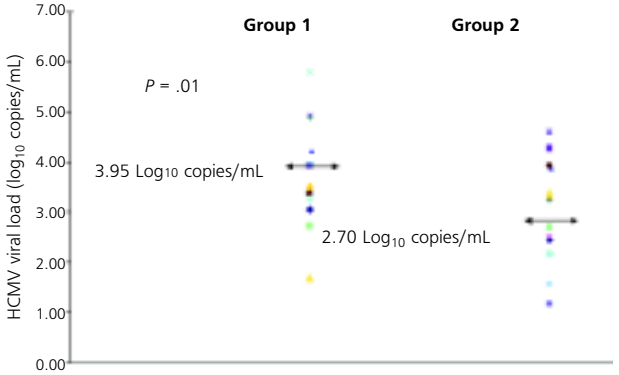

Figure 1C. HCMV, Human *Cyomegalovirus* viral load at the moment of the highest DNAemia test value in treated vs untreated patients.

observed in 2 patients who exhibited a high viral load (> 3 $\log_{10}$ copies/mL [n = 1] and 4 log10 copies/mL [n = 1], respectively). Full-length sequencing of the UL97 and UL54 genes to reveal related ganciclovir-resistant mutations indicated 3 new UL97 mutations in 1 of those 2 patients. Detected in 3 sequential samples, those new mutations (Arg 112 His, Arg 164 His, and Arg 285 Ser) were located within the N-terminal domain of UL97 phosphotransferase. Their potential participation in ganciclovir resistance is presently under investigation. However, because mutations 112 and 164 are located outside the conserved domains, Arg 112 His, Arg 164 His, and Arg 285 Ser are likely to be polymorphisms. None of the patients from group 2 showed a recurrence of HCMV DNAemia during the follow-up period.

Discussion

Quantitative real-time PCR seems to be the most effective tool for monitoring renal transplant recipients [24]. However, in low-risk patients (ie, HCMV-seropositive recipients), whether preemptive treatment or symptom-triggered treatment is the most effective method has not been determined. In cases of preemptive treatment, the optimum threshold value has not been well established. Different threshold values (ie, 5000, 7000, or 10,000 copies/mL) have been evaluated [7, 26, 27]. We defined the threshold value in HCMV-seropositive renal transplant recipients as 10,000 copies/mL [23].

However, the monitoring of renal transplant recipients differs from that of patients who have undergone OLT. Several studies have been conducted in the latter population, but only a very few investigations have involved pretransplantation HCMV-seropositive recipients. To our knowledge, our study is the first to address HCMV monitoring with quantitative real-time PCR using DNA that was automatically extracted from whole blood with a MagNA Pure instrument (Roche Molecular Biochemicals) in HCMV-seropositive OLT recipients who received either a seropositive or seronegative transplants.

In our study, 42 of 70 (60%) OLT patients were HCMV seropositive before they underwent liver transplantation. Twenty-seven (64%) of those patients experienced at least 1 incident of HCMV DNAemia. This incidence is much higher than that reported by other investigators: Seehofer and coworkers [18] showed a very low incidence (18.6%) of HCMV-related DNAemia in OLT patients, regardless of serologic HCMV status before transplantation. Singh and coworkers [28] detected HCMV infection in only 32.5% of HCMV-seropositive liver transplant recipients. Those different rates might be due to the various techniques of HCMV detection used to analyze blood; ie, pp65 antigenemic testing versus quantitative real-time PCR. Moreover, the patients in our study did not receive anti-HCMV prophylaxis. This shows that, despite anti-HCMV prophylaxis, 34% of patients did not experience HCMV DNAemia during the whole follow-up.

Even though the incidence of HCMV infection in R+ patients was high, only 44% of the infected patients (n = 12) showed clinical and/or biologic

abnormalities compatible with HCMV disease. Those patients were treated with IV ganciclovir for a mean of 15 days (group 1), and the patients in group 2 (n = 15) were not so treated. Therefore, more than half of the infected patients (56%) who were not treated did not demonstrate any adverse effects.

In HCMV-infected patients, we prospectively monitored the biochemical and hematologic markers at the time of transplantation, at every 2 weeks after transplantation until day 90, and thereafter at 3-week to 4-week intervals until day 180, as well as at the time of the first positive HCMV DNAemia test result. We compared the HCMV viral load in the treated versus the untreated patients at the time of the first positive test and that of the highest DNAemia. The ALT, AST, γGT, and bilirubin levels were higher in group 1 than in group 2; however, those differences were not significant. The AP levels were higher in group 1 than in group 2 ($P = .001$), and the hemoglobin levels were lower in group 1 than in group 2 ($P = .04$). Other factors were similar in both groups. Virologic parameters did not show a significant difference regarding the time between transplantation and the occurrence of the first DNAemia episode or the highest DNAemia value. At the time of the first HCMV DNAemia test, the viral load was statistically different in treated versus untreated patients ($P = .01$). The highest viral load was also statistically different between the 2 groups ($P = .01$). The results of multivariate analyses showed that the only independent predictive factor associated with the treatment of the first HCMV DNAemia episode was the AP level.

A good response was shown in the decrease and eradication of HCMV in blood from those in group 1. Two patients who experienced a second recurrence of HCMV DNAemia were cleared of that episode after treatment. Untreated patients were cleared of their HCMV as shown by the negative real-time PCR results, and none of those individuals experienced a second HCMV reactivation or demonstrated any adverse effects regarding liver and hematologic function; ie, they did not show HCMV disease.

Our study has shown that HCMV-seropositive patients who have undergone OLT can benefit from the absence of HCMV prophylaxis after liver transplantation. Moreover, HCMV infection can be monitored by a regular and standardized follow-up of clinical and biologic markers, and HCMV DNAemia can be monitored by quantitative real-time PCR. The initiation of antiviral treatment can be delayed until the development of clinical or biologic symptoms or until a high viral load ($> 4 \log_{10}$ copies/mL) has been identified. However, a high viral load does not affect the response to treatment: Three patients with a high viral load recovered spontaneously from their HCMV, although 1 patient with a viral load of between 3 and 4 $\log_{10}$ copies/mL did not.

Our results confirm that HCMV viremic patients who have a low viral load ($< 4 \log_{10}$/mL) and no clinical or biologic symptoms may benefit from an absence of treatment without impairment of their liver function. Moreover, in patients with HCMV viremia, an alkaline phosphatase level > 150 IU/mL may be a biologic marker for the initiation of treatment.

In conclusion, we propose these guidelines for the follow-up of HCMV-seropositive patients who have undergone OLT: the absence of anti-HCMV prophylaxis, monitoring with quantitative real-time PCR, and a survey of clinical and biologic manifestations of disease. Anti-HCMV treatment can be initiated in patients with HCMV DNAemia that is associated with clinical symptoms or an HCMV DNAemia viral load of $> 4 \log_{10}$ copies/mL or, perhaps most importantly, when HCMV DNAemia is associated with an alkaline phosphatase level of > 150 IU/L.

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