

## Diagnosis of Cytomegalovirus in Immunocompromised Persons

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### Abstract

Cytomegalovirus is the leading viral cause of congenital disease, often producing serious neurological deficits. CMV attacks the developing central nervous system (CNS) resulting in serious brain disorders that include microencephaly, epilepsy, deafness, microgyria, mental retardation, sensory loss, motor problems, and psychiatric disturbances. In addition, CMV is a clinically important opportunistic virus that can lead to serious neurological disease in AIDS patients. The present application addresses basic mechanisms of viral spread into the brain, and once in the brain, spread by intracellular transport or extracellular diffusion to other brain cells. The hypothesis that CMV can be spread through axonal transport will be studied with *in vitro* and *in vivo* models. Although CMV appears to have no absolute host cell preference in the brain, the hypothesis that CMV shows relative cellular preferences will be tested in living brain slices at different developmental ages. Using a mouse model of immunosuppression, parallel to AIDS, we will test the hypothesis that cell-mediated immunity protects neurons *in vivo* from CMV proliferation. Neuronal activity plays an important role in establishing the correct circuitry during brain development. The hypothesis that early infection by CMV can generate disturbances in the electrophysiological activity of developing neurons will be tested with whole cell patch clamp recording using current and voltage clamp electrophysiology, and with calcium digital imaging, using primary mouse neuron cultures and brain slices. Virus mediated changes in intracellular ion levels, ion currents, transmitter responses, and membrane properties will be compared in CMV infected and control cells.

**Keywords:** CMV Infected, Immunosuppression, Immunosuppression, Microencephaly

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### Introduction

Human cytomegalovirus (HCMV), a double-stranded DNA virus in the herpes virus family, is a ubiquitous virus that infects greater than 40–60% of the general population and up to 100% within some subpopulations and/or geographic areas [1]. HCMV has a complex pathobiology because infection of immunocompetent individuals is rarely associated with severe clinical symptoms and in most cases is simply asymptomatic, whereas HCMV infections can cause a wide range of severe diseases, including mononucleosis, mental retardation, deafness, chorioretinitis, and fatal diseases, such as interstitial pneumonia and disseminated virus infections in immunocompromised hosts [1]. As with other herpes viruses, HCMV is thought to establish latent or persistent infections. Reactivation of this infection is frequently encountered during pregnancy and in organ transplant and acquired immune deficiency syndrome (AIDS) patients [1].

In addition, HCMV has been implicated as a co-etiological agent in cervical cancer [2] and has been found associated with a wide range of other tumors [1]. More recently, HCMV has also been shown to be epidemiologically linked to restenosis [3–5] and atherosclerosis [5, 6]. The severity of these HCMV-associate diseases warrants an

accurate ability to detect and diagnose persons with HCMV, especially because of the clinical availability of the anti-HCMV agents, ganciclovir and foscarnet, which have been used successfully to treat patients with HCMV viremia. Early and accurate detection is needed especially in cases in which infected individuals are at risk of complications arising from HCMV infection; for example, in patients undergoing organ transplants or angioplasty, expectant mothers, or AIDS patients. The detection or diagnosis of HCMV infection in the laboratory can be achieved by a number of different approaches, including both classical methods (i.e., virus isolation, some serologic tests, and the search for cytomegalic inclusions in histological sections) and more modern methods (i.e., Western blot analysis, enzyme linked immunosorbent assay [ELISA], nucleic acid hybridization, and the polymerase chain reaction [PCR]).

The appropriate method to use depends on the type of technique, used to collect the specimens and the type of answer that is desired. Because of limitations, we discuss only the modern immunological techniques for detecting HCMV in the laboratory setting. The focus of this discussion is the common immunological methods for the detection of HCMV, including ELISA, immunohistology (immunofluorescence and immunoperoxidase), and



Western blot analysis as it pertains to the use of immunological reagents, antibodies. Since other methods exist [7–10], we also included a brief synopsis of these additional methods and provided some references for the reader's convenience to allow for a more focused understanding of these additional techniques.

## Materials and Methods

### ELISA

As a source of HCMV antigen and control extracts,  $2 \times 850\text{-cm}^2$  roller-bottle (Corning, Corning, NY) cultures of human fibroblasts are needed: (1) a roller bottle with 100% of the cells showing cytopathic effects (CPE), and (2) a roller bottle of uninfected cells as a control. 0.15 M glycine buffer (pH 9.0), 100 ml is needed. Microtiter plates (Nunc, Corning, or Falcon Elisa Plates [Fisher]).

1–2 L of phosphate-buffered saline (PBS). Blocking solution: 0.1 M Tris-HCl (Boehringer, Mannheim, Indianapolis, IN; pH 8.0), 2% protease free bovine serum albumin (BSA; Boehringer Mannheim) and 0.1% thimersol (Sigma, St. Louis, MO; ~50 ml is needed). Human serum sample(s). 100 ml of PBS containing 0.1% Triton X-100 (Sigma). Alkaline phosphatase (AP)-conjugated or horseradish peroxidase (HRP)-conjugated anti-human IgG or IgM secondary antibody (i.e., Sigma, Santa Cruz, Santa Cruz, CA, or Vector Laboratories, Burlingame, CA). The colorimetric substrate for AP-conjugated secondary antibodies, is p-nitrophenyl phosphate (NPP; Sigma), a solution containing 3 mM NPP, 0.05 M  $\text{Na}_2\text{CO}_3$  (Mallinckrodt, Paris, KY), and 0.05 mM  $\text{MgCl}_2$  diluted in  $\text{dH}_2\text{O}$  is needed (10 ml is needed for each 96-well microtiter plate used; this solution should be stored at  $4^\circ\text{C}$ ). For HRP-conjugated secondary antibodies, the substrate is 3,3',5,5'-tetramethylbenzidine (TMB), two solutions should be made: Solution A (urea hydrogen peroxide; Sigma): 0.054 g urea hydrogen peroxide, diluted in 100 ml of 0.1 M sodium citrate (adjusted to pH 5.0 with  $\text{H}_3\text{PO}_4$ ; Fisher) Solution B (the colorimetric substrate): 30 mg of TMB, dissolved in 1 ml of DMSO and then diluted to a final concentration of 0.3 mg/ml in a 100-ml solution containing 10% glycerol (10 ml), 30% methanol (30 ml), and  $\text{dH}_2\text{O}$  (59 ml). This solution should be stored in the dark. 0.5 M Ethylenedinitrilo tetraacetic acid (EDTA). ELISA plate reader. These reagents are generally very stable and can be stored for months.

### Immunofluorescence and Immunoperoxidase Assays

A freshly prepared paraformaldehyde solution containing 3% paraformaldehyde (3 g), 2% sucrose (2 g) in 100 ml of PBS. The paraformaldehyde solution must be neutralized with 2 N NaOH to a pH 7.0 and heated to  $60^\circ\text{C}$  to allow it to go into solution. Care should be exercised when heating this product because formaldehyde is a carcinogen (this step should be performed in a chemical hood). Wash buffer (PBS). 10% normal goat serum (Accurate Chemical and Scientific Corporation, Westbury, NY; bovine serum albumin [BSA] or the serum from the species of animal in which the primary or secondary antibody was prepared) diluted in PBS. Permeabilization buffer (0.5% Triton X-100 and 300 mM sucrose diluted in

PBS). Make up 100 ml of this solution and store at  $4^\circ\text{C}$ . An appropriate primary antibody targeting the HCMV gene product of interest; some are available commercially (e.g., Vancouver Biotech, British Columbia, Canada; Rumbaugh-Goodwin Institute for Cancer Research, Plantation, FL; Dupont, Boston, MA), but most have been developed in the laboratories of individual researchers. The appropriate fluorescently-conjugated (usually fluorescein isothiocyanate [FITC]) secondary antibody (e.g., Sigma, Vector Laboratories, etc.), 50% glycerol (Fisher) in PBS and a fluorescent microscope.

### Western Blot Analysis

The detection of specific HCMV antigens can also be accomplished by gel electrophoresis and Western blot analysis. This is usually a very specific and sensitive assay that excels in its ability to detect specific proteins of interest (For further details of this technique, see ref. 7.) Briefly, this technique involves the resolution of different protein species by molecular weight and then the transfer of these proteins to a solid matrix such as nitrocellulose. Then, in a principle analogous to the indirect ELISA, a primary antibody is incubated first, followed by incubation of a secondary HRP- or AP conjugated antibody and then developed using a colorimetric reaction in which the specific proteins/antigens of interest are identified by a band on the nitrocellulose matrix. We have only included a brief description of this technique because of its use of immunological reagents and its similarity in principle to the other techniques. To perform a Western blot, use the following procedure: Infected cell lysates or clinically infected tissue for gel electrophoresis and Western blot analysis are harvested in a SDS-PAGE sample buffer, boiled, and then either used immediately or stored at  $-20^\circ\text{C}$ .

Samples are electrophoresed on a 5–15% SDS-PAGE gel, depending on the molecular weight of the protein(s) of interest along with a molecular weight marker. Equal protein amounts are always added to each well. We have not included the detailed information about preparing a gel for electrophoresis or running a protein gel because of the space limitations within this chapter (for more information, see ref. 7). Instead, we have focused on the actual Western blot protocol.

The proteins are transferred to nitrocellulose (Immobilon-P; Millipore). The time used to transfer the proteins varies on the size of the gel, the speed with which it is run, and the molecular weight of the proteins of interest (smaller proteins transfer faster).

For instance, it is possible to transfer nearly all of the proteins in a mini-gel to a nitrocellulose membrane at 14–20 V overnight or at 100 V for 1–2 h.

For accurate measurement of the success of a protein transfer, the reversible protein stain Ponceau S can be used. The nitrocellulose blot(s) is incubated for 5 min at room temperature with enough Ponceau S to barely cover the surface of the nitrocellulose, and then carefully washed in  $\text{dH}_2\text{O}$  for 1–2 min. During this time, the background red color will slowly disappear leaving only the major protein species. At this point, the molecular-weight markers should be marked on the membrane with indelible ink;

then, if needed, the membrane can be wrapped in plastic wrap and xeroxed for a permanent record of the protein loading for each lane.

Next, the blots are incubated in a blocking solution for 1 h. The blots are then incubated with an anti-HCMV primary antibody, diluted 1/100–1/5000 in the blocking solution (this dilution will generally have to be determined for each antibody used) for 1 h at room temperature (this time can be increased, depending on the strength of the signal to as long as overnight) with gentle rocking.

The blots are washed three times for 10–15 min each in the washing solution with gentle rocking. Incubate the blots for 1 h with the appropriately conjugated secondary antibody (we prefer an HRP-conjugated antibody) with gentle rocking. Wash the blots 3–5 times in the washing solution for 10 min each and then two times in PBS with rocking. The blots are then incubated with the developing agent and developed according to the Amersham Life Sciences ECL protocol.

#### Detection of HCMV Infection by PCR

The use of PCR offers two main advantages over many other techniques: sensitivity and speed. In this assay, a specific segment of DNA (i.e., cellular or viral) is amplified by a heat stable DNA dependent DNA polymerase, such as Taq or Vent polymerase by using specific oligonucleotide primers that hybridize to the complementary DNA (cDNA) strands flanking the specific DNA fragment of interest, in this case a specific HCMV viral sequence (for a more complete introduction to PCR, see ref. 7). The amplified DNA fragment(s) can be amplified up to one million-fold and can be identified by agarose gel electrophoresis and, if needed by Southern blot analysis as well [7].

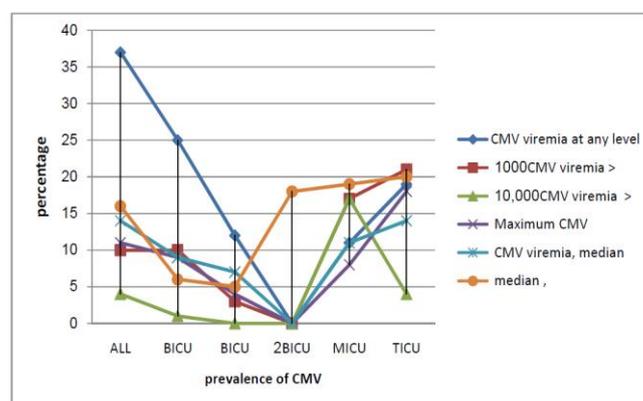
PCR has been shown to detect HCMV in the urine of newborns, in the blood of AIDS patients, in organ transplant patients, in Kaposi's sarcoma specimens, in the lungs of patients with interstitial pneumonitis, in the arterial walls of patients suffering from atherosclerosis, and in cervicovaginal cells (see ref. 8 and the references within). The sensitivity of this assay has been estimated to be at the level of 1 viral genome per 40,000 cells [8]. The superb sensitivity and specificity of PCR assay make it one of the best methods in the clinical laboratory for detecting HCMV in a great variety of clinical samples.

#### Results

A total of 221 patients were initially screened for inclusion in the study, and 101 were excluded on the basis of: a negative CMV serology ( $n = 78$ ), death or discharge within 72 hours of admission ( $n = 8$ ), inability to obtain informed consent ( $n = 9$ ), or other miscellaneous reason(s) ( $n = 6$ ), leaving 120 patients who comprised the study population. The characteristics of the study population stratified by ICU are shown in Table 1. Forty patients were enrolled in each the MICU and TICU and 20 patients each in the BICU and CICU. The primary composite endpoint of continued hospitalization or death by 30 days occurred in 45 of 120 patients (38%).

#### Incidence and quantization of CMV reactivation (viremia)

The incidence of CMV viremia stratified by ICU is shown in Figure 1. A total of 1,954 samples were tested from the 120 enrolled patients, with a median and range of 11 (1–89) samples tested per patient. The cumulative incidence of CMV viremia at any level (panel A) and  $>1,000$  copies/ml (panel B) stratified by ICU, and for the entire cohort (panel C) is shown in Table 1. The cumulative incidence estimate of CMV viremia at any level was 33% (39 of 120). Among patients in whom viremia ever developed, 37 of 39 (95%) did so within the first 30 days after admission to the ICU, and half within the first 12 days (range 3–57 days to first detectable viremia). The cumulative incidence estimate of CMV viremia  $> 1,000$  copies/ml was 20% (24 of 120), occurring at a median of 26 days (range 9–56). The 95% CI for the cumulative incidence estimates of CMV viremia at 30 days at either any level or  $> 1,000$  copies/ml for the BICU, CICU, MICU, and TICU were: 0.23–0.67, 0–0.31, 0.12–0.38, 0.22–0.53 and 0–0.23, 0–0.15, 0.04–0.26, and 0.08–0.32, respectively.



**Figure 1.** Cumulative incidence of CMV viremia at any level stratified by ICU.

#### Risk factors for CMV reactivation

Multivariable logistic regression analysis of factors associated with CMV viremia at any level is shown in Table 1. In multivariable models, male gender was associated with an increased risk for CMV reactivation. The APACHE II score at admission was not associated with an increased risk of subsequent CMV reactivation. The results were similar when a CMV viremia endpoint of  $>1,000$  copies/ml was used, except that the baseline variables of ventilator use (adjusted OR 8.5 [1.1–66.5],  $p = 0.04$ ) and receipt of a transfusion (adjusted OR 6.7 [1.1–42.7],  $p = 0.05$ ) were associated with an increased risk for CMV reactivation at that level (data not shown).

#### Risk factors for death or continued hospitalization by 30 days

Table 2 shows the raw data for: discharge, death, continued hospitalization, and CMV reactivation status of the cohort by day 7, 10, 15, 20, and 30 after admission to the intensive care unit. Table 1 shows the logistic regression univariable and multivariable analysis of factors associated with the composite endpoint of death or continued hospitalization by 30 days after admission to the ICU. Even when adjusted for other significant baseline or time-dependent variables, CMV reactivation assessed in

any one of four ways (viremia at any level, >1,000 copies/ml, maximum viremia in log<sub>10</sub> copies/ml, or average AUC) was independently associated with death or continued hospitalization by 30 days. Furthermore, there was a quantitative association, such that the greater the amount of CMV reactivation the greater the risk for continued hospitalization or death by 30 days.

A similar association between CMV reactivation and death or continued hospitalization by the earlier time-point of 15 days was evident (adjusted HR [95% confidence interval] for viremia at any level; 6.1 [1.7-21.7],  $p < 0.01$ , maximum viremia in log<sub>10</sub> copies/ml; 2.1 [1.2-3.7],  $p < 0.01$ , or average AUC; 2.6 [1.1-6.2],  $p = 0.03$ ). Table 2 shows the predicted probability (with 95% CIs) of death or continued hospitalization by 30 days as a function of the average CMV AUC based on a logistic regression model. Each log increase in the average CMV AUC was associated with a 14% increase in the probability of death or continued hospitalization by 30 days. A similar analysis but using as the composite endpoint death or ICU (rather than total) hospitalization by 30 days yielded similar results: each of the CMV variables remained associated with death or ICU hospitalization by 30 days, with adjusted odds ratios and 95% CIs for CMV viremia at any level (5.7 [2.1-15.6],  $p < 0.001$ ), >1,000 copies/ml (4.6 [1.2-17.4],  $p = 0.02$ ), each log<sub>10</sub> maximum CMV (1.7 [1.2-2.4],  $p = 0.002$ ), and average AUC of CMV (2.0 [1.3-3.1],  $p = 0.003$ ), respectively. As reported in prior studies, we confirmed that development of a major infection (nosocomial bacteremia or pneumonia) was associated with an increased hospital LOS (adjusted OR 3.0, 95% CI, 1.1-8.4,  $p = 0.04$ ). The association between CMV and death or continued hospitalization by 30 days after admission to the ICU remained significant when the analysis was restricted to the MICU and TICU cohorts only, with adjusted odds ratios and 95% CIs for CMV viremia at any level (7.3 [2.3-22.9],  $p < 0.0001$ ), >1,000 copies/ml (32.4 [5.8-18.3],  $p < 0.0001$ ), each log<sub>10</sub> maximum CMV (2.1 [1.5-3.0],  $p < 0.0001$ ), and average AUC of CMV (2.7 [1.6-4.3],  $p < 0.0001$ ), respectively.

#### **Risk factors for increased length of hospitalization**

We used the variable “seven-day CMV moving average” to model the short-term effects of higher CMV viral load on the odds of staying longer in the hospital. In addition, we used the “average CMV AUC” to model the long-term effects; i.e., the lasting effects of previous high viral loads on length of hospitalization. Table 2 shows that overall, a higher CMV moving average over the previous seven days or average CMV AUC was associated with an increased hospital LOS. For example, for each log<sub>10</sub> copy/ml increase in viral load over the previous seven days, there was a 5.1-fold increased odd of being hospitalized for more than 14 days; similarly, for each log<sub>10</sub> increase in viral load seven-day moving average, there was a 2.8-fold increased odds of being hospitalized for more than 28 days. This association did not remain significant for the more extreme LOS (i.e., for LOS greater than either 42 or 56 days). The average CMV AUC was also associated with an increased odds of continued hospitalization,

regardless of when during the hospital stay this parameter was assessed (data not shown).

To assess the impact of CMV reactivation on length of stay in a group who was uniformly monitored for CMV reactivation, we performed a landmark analysis and assessed the cumulative incidence of time to discharge among the 35 patients who were still hospitalized by day 30 after admission. Patients were categorized as CMV reactivators if they tested positive by PCR prior to day 30. According to Figure 1 the hazard of discharge is significantly greater in non-reactivators as compared to reactivators ( $p$ -value = 0.03 by log-rank test). The median length of stay after day 30 in reactivators ( $n=21$ ) was 24 days (range 3-64) compared to 10 days (range 1-151) in non-reactivators ( $n=14$ ).

#### **Discussion**

Using a prospective, blinded study design and rigorous statistical analyses in a broad range of immunocompetent patients with critical illness, we demonstrated that reactivation of CMV occurs frequently and is independently and quantitatively associated with a clinically-relevant endpoint of continued hospitalization or death by 30 days after admission to the ICU. Thus, we have identified a novel and potentially modifiable risk factor for death or prolonged hospitalization in critically-ill patients.

Given the number, complexity, potential bi-directional relationships between CMV and other variables analyzed, and the time-varying nature of the endpoints, we used a variety of statistical methods to comprehensively assess the relationship between CMV and adverse clinical outcomes. These included use of partial proportional odds models, use of a novel parameter of seven-day moving average of CMV viral load throughout the hospital stay, and use of a composite endpoint of death or continued hospitalization by 30 days. In particular, use of the composite endpoint was objective, clinically relevant and one that could be used as a primary endpoint in subsequent interventional studies of CMV prevention in this setting. Furthermore, the composite endpoint (rather than use of only length of stay alone) was used to reduce the potential impact that early deaths might have on assessment of the relationship between CMV reactivation and LOS. Similarly, use of the partial proportional odds models allowed us to control for the observed relationship between length of stay and onset of CMV reactivation, thereby allowing the relationship of CMV reactivation and subsequent LOS to be assessed throughout the hospital stay. In addition, given the concern that longer LOS would lead to a greater opportunity to detect CMV reactivation (and thus potentially lead to a spurious association between CMV reactivation and LOS), we performed a landmark analysis among those who were hospitalized for at least 30 days (a time-point by which 95% of those who ultimately ever reactivated CMV had done so, and also a subset who all had a uniform duration of monitoring for CMV). And, as in the previous analyses, CMV reactivation was associated with longer durations of subsequent hospitalization compared to those who did not reactivate by day 30 (Table 2). The association between CMV reactivation and prolonged

hospitalization or death remained robust throughout all of the analyses. Thus, our data are consistent with the possibility that CMV reactivation is causally related to prolongation of hospital stay in this clinical setting and this contention is also supported by animal studies.

was simply a surrogate marker of illness severity. The only definitive means of differentiating between a role of CMV as a cause versus marker for adverse clinical outcomes is by means of a randomized controlled trial of antiviral prophylaxis in this clinical setting.

**Table 1.** CMV reactivation as assessed by PCR

CMV Variable	ALL	BICU	CICU	MICU	TICU
	n=120	n=20	n=20	n=40	n=40
CMV viremia at any level, n (%)	39 (33)	11 (55)	3 (15)	10 (25)	15 (38)
CMV viremia >1000 copies/ml, n (%)	24 (20)	9 (45)	1 (5)	6 (15)	8 (20)
CMV viremia >10,000 copies/ml, n (%)	11 (9)	4 (20)	0 (0)	4 (10)	3 (8)
Maximum CMV load (log10 PCR copies), median (range)	3.3 (1.8-5.5)	3.9 (2.5-5.5)	2.4 (1.8-3.7)	3.4 (2.3-4.8)	3.1 (2.1-4.5)
Days to first detectable CMV viremia, median (range)	12 (3-57)	19 (7-57)	15 (9-21)	8 (3-13)	11 (3-21)
Duration of shedding in days, median (range)	17 (2-45)	20 (4-45)	4 (2-17)	18 (4-38)	14 (2-32)

**Table 2.** Raw data for status of hospitalization, mortality, and CMV reactivation by day after admission.

	Index Day				
	Day 7	Day 10	Day 15	Day 20	Day 30
Discharged before	22	33	46	61	75
Died before	3	7	9	9	10
Continued hospitalization on	96	80	65	50	35
<b>Among patients still hospitalized on index day</b>					
Never CMV reactivated	58	40	32	12	13
Reactivated before	12	14	23	21	22
Reactivated after	29	23	11	8	3
CMV 7-day moving average at index day in log copies/ml (median, range)	0/2 (0.1-2.3)	0.8 (0.2-2.5)	0.5 (0-2.3)	1.1 (0-3.4)	2.2 (0-4.1)
P-value*	<0.0001	<0.0001	0.0001	<0.0001	
CMV average AUC at index day in log copies/ml (median, range)	1.2 (0.1-2.1)	1.4 (0.6-2.6)	1.3 (0.3-3.1)	1.6 (0.3-3.6)	2.1 (0.9-3.5)
P-value#	0.001	<0.0001	0.002	<0.0001	

\* P-value compares CMV 7-day moving average at index day with 7-day moving average at day 30 adjusted for intra-subject correlation using GEE.

# P-value compares average CMV AUC at index day with average AUC at day 30 adjusted for intra-subject correlation using GEE.

However, we are careful to emphasize that an observational study design cannot establish causality, also the data presented here are also consistent with the possibility that CMV reactivation is simply a marker (rather than determinant) for prolonged hospital stay. Importantly, we did not find an association between severity of illness (as assessed by the APACHE score) and risk of CMV reactivation, thereby diminishing the likelihood that CMV reactivation

Given the major importance of the clinical problem, the availability of generally safe and well-tolerated antiviral agents with activity against CMV, combined with the data regarding CMV incidence and endpoint estimates generated in this study, we feel that a randomized placebo-controlled trial of antiviral prophylaxis is both warranted and feasible, and should be a priority among

studies to improve the outcomes of patients with critical illness.

The mechanism(s) underlying the observed association between CMV and adverse clinical outcomes are not defined in the present study. One possibility is direct CMV pathogenicity and this has previously been reported in the setting of otherwise immunocompetent patients with critical illness, but appears to be uncommon.

Another possibility is that one or more CMV indirect effects are responsible for the observed association between CMV reactivation and adverse clinical outcomes. CMV-mediated immunosuppression leading to an increased risk for secondary infections and CMV-mediated lung injury are the most plausible mechanisms in this clinical setting. In support of these possibilities are *in vitro* and animal model experimental data, clinical observational studies and the demonstration that antiviral therapy reduces these effects in animal models and in controlled clinical trials in certain patient populations. Larger prospective studies that include laboratory investigations will be necessary to define the mechanism(s) underlying the association of CMV reactivation with adverse clinical outcomes in patients with critical illness.

There were several strengths of the present study, including the prospective, blinded study design, inclusion of a broad range of critically-ill patients, use of quantitative CMV assessments, and the use of comprehensive statistical analyses with an adequate number and frequency of clinically-relevant endpoints. This is the largest study conducted to date and the results are statistically robust. It is reassuring that factors previously reported to be associated with increased LOS (bacteremia, pneumonia) were confirmed to be associated with LOS in the present study. We also acknowledge potential limitations.

Monitoring for CMV reactivation was not performed in discharged patients, and while we think it is unlikely, it is possible that some discharged patients may have first reactivated CMV after hospital discharge. Although this would not have altered the statistical assessment of the association between CMV and LOS, it would make it more difficult to conclude that CMV was having a biologically significant impact in this clinical setting. There is also the potential concern that the association between CMV reactivation and prolonged hospital stay could, in part, be related to a greater opportunity to detect CMV reactivation in those with longer hospital stays (i.e., "circular reasoning"). However, the known biological time-lag of CMV effects in other settings, the quantitative nature of the association demonstrated in the present study, and the consistent finding of the association between CMV reactivation and prolonged LOS in the landmark analysis and partial proportional odds models (both of which directly addressed the time-dependent nature of CMV reactivation) all support the contention that CMV reactivation was associated with prolongation of hospital stay rather than a spurious finding. We are careful to emphasize that our study design (or any observational study design) cannot prove causality between CMV and adverse clinical outcomes in this setting. Rather, we consider these results to be hypothesis-generating and provide useful background

data, which when combined with prior investigations, provide the rationale for performing definitive interventional studies. Even though a strong association between CMV reactivation and prolonged length of stay was identified, the mechanism(s) underlying this association could not be defined in this study. And, not all variables previously reported to be associated with an increased LOS were examined in the present study.

In summary, we have demonstrated an independent and quantitative association between CMV viral load and prolonged length of stay in a broad range of immunocompetent patients with critical illness. These findings, combined with data from prior investigations, provide a strong rationale for a randomized controlled trial of antiviral prophylaxis in this clinical setting.

### Conclusion

Numerous methods and approaches can be used to diagnose and detect HCMV infection; each has its own advantages. The optimal and appropriate method(s) to be used frequently depend(s) on the nature of the clinical sample collected and the clinical manifestation at the time a sample is collected. In general, virus isolation and immunocytochemistry are good choices for the defined and rapid diagnosis of HCMV infection in biopsied or autopsied specimens and exfoliated cells. The ELISA also offers rapid and accurate detection of HCMV infections when anti-HCMV antiserum is available; however, it may not always give accurate results when patients become immunotolerant to HCMV. Thus, it is always a good idea to use a combination of tests to detect HCMV infections. In addition, *in situ* hybridization can also be an effective tool for confirmation of HCMV infection. Nevertheless, it is important to determine the technique or combination of techniques that allows one to address the specific questions that need to be answered. Most of the techniques described above can be used very effectively in both clinical and molecular virology laboratories to detect and characterize the consequences of HCMV infection accurately.

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