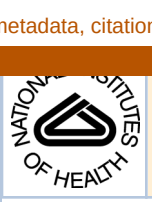



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# Reactivation of Herpes Simplex Virus Type 1 is Associated with Cytomegalovirus and Age

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## Abstract

Recent studies have shown that Cytomegalovirus (CMV) may be an emerging marker of immunosenescence. CMV can affect the immune system by directly infecting leukocytes and hematopoietic cells or by eliciting an expansion of oligoclonal CD8+ T cells/contraction of the naïve T cell compartment that may reduce the host's ability to fight other pathogens. To investigate further CMV-associated changes in immunity, a study was conducted with 1,454 adults (ages 25–91) to determine the association between CMV and reactivation of another latent herpesvirus, Herpes simplex virus type 1 (HSV-1), as indexed by antibody titers. Elevated antibody titers to latent HSV-1 were significantly associated with both CMV seropositivity and high CMV antibody levels. Evaluation by specific age groups (<45, 45–64, and 65+ years old) revealed that this association was detectable early in life (<45 years of age). Increases in HSV-1 antibodies by age occurred in CMV seropositive individuals but not CMV seronegative subjects. Within CMV seropositive subjects, increases in HSV-1 antibodies by age were only found in individuals with low CMV antibody levels as those with high CMV antibodies already exhibited elevated HSV-1 antibodies. These associations remained significant after accounting for body mass index, gender, and socioeconomic status. These results suggest that CMV can influence the immune response to another pathogen and support the concept that CMV may accelerate immunosenescence.

### Keywords

herpesvirus; Herpes simplex virus (HSV-1); Cytomegalovirus (CMV); aging; immunosenescence

## INTRODUCTION

Herpesviruses are a clinically important group of viruses that can cause a variety of diseases. Herpesvirus infections are usually characterized by an acute phase associated with minor morbidity and mortality followed by a chronic latent phase reflecting a balance between viral replication and the host immune response. In previously infected (seropositive)

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individuals, antiviral IgG antibodies are always present and are tightly regulated by the immune system due to control over viral reactivation by cytotoxic T cells [Okano et al., 1988; Wallace et al., 1999; Babel et al., 2009]. Therefore, levels of herpesvirus antibodies in healthy individuals are quite stable.

However, a dysfunction of the immune system permits viral reactivation and leads to increased levels of antiviral antibodies. For instance, age-related declines in cellular immunity are associated with increased herpesvirus antibodies [Glaser et al., 1985; Musiani et al., 1988; Weymouth et al., 1990]. In addition to elevated antibodies, increased viral gene transcription and increased viral DNA were subsequently found in a study of herpesvirus reactivation in older adults [Stowe et al., 2007]; viral DNA and viral gene transcription in samples from younger adults in this same study were at or below detectable levels which corresponded with low antiviral antibody titers.

Cytotoxic T cell function may also be impaired due to stress, resulting in replication and increased production of antiviral antibodies [Glaser et al., 1987; Glaser et al., 1991; Glaser et al., 1993; Glaser et al., 1994]. Studies of astronauts have demonstrated increased herpesvirus antibodies, increased viral gene transcription, and increased viral load as a result of stress and spaceflight [Mehta et al., 2000, 2004; Pierson et al., 2005; Stowe et al., 2001a, 2001b, 2011]. Importantly, these studies demonstrate a direct correlation between elevated antiviral antibodies and detection of viral DNA. Thus, levels of antiviral antibodies have been used as surrogate markers of cellular immune function [McDade et al., 2000; Stowe et al., 2001a, 2001b; Glaser and Kiecolt-Glaser, 2005; Dowd et al., 2011].

Recent studies have suggested that infection with Cytomegalovirus (CMV), a member of the herpesvirus family, may be associated with adverse health in the elderly [Bennett et al., 2011]. CMV infection typically occurs asymptomatically during childhood; by age eleven approximately 36% of individuals are CMV seropositive and this increases to approximately 60% by age forty [Staras et al., 2006]. In CMV seropositive individuals, a progressive accumulation of CMV-specific CD8+ T cells occurs over time that results in a significant portion of the total CD8+ T cell pool in older adults; the frequency of CMV-specific T cells may reach 25% or more [Gillespie et al., 2000; Khan et al., 2002]. It has been proposed that such expansions may reduce the immune response to antigenic challenge via a reduction in the naïve T cell compartment [Pawelec et al., 2004]. Subsequently, it was shown that T cell responses to Epstein-Barr virus (EBV) were impaired in subjects infected with CMV [Khan et al., 2004].

Because of the growing interest on age and latent herpesvirus infections [Bennett et al., 2011; Simanek et al., 2011], samples were collected from a large number of subjects to examine associations between age, CMV infection, and Herpes simplex virus type 1 (HSV-1) reactivation (indexed by antibody titers). It was hypothesized that CMV seropositive subjects would have impaired control of HSV-1 leading to reactivation of latent HSV-1. Furthermore, it was hypothesized that elevated CMV antibodies would positively correlate with high HSV-1 titers.

## METHODS

### Subjects

Respondents for this study come from the Texas City Stress and Health Study, an ongoing assessment of risk, coping, stress, and health in a tri-ethnic community living in Texas City, TX [Peek et al., 2009]. The sample was generated through a census of Hispanic households and a simple random sample of non-Hispanic households. Three ethnic strata were selected: Mexican Americans aged 25–64, Mexican Americans aged 65 and over, and non-Hispanics.

Housing units (HUs) were selected in each stratum, including all Hispanic HUs and 1 in 8 non-Hispanic HUs. Finally, one adult per household was randomly selected among Mexican Americans and among non-Hispanics, and all Mexican Americans aged 65 and over. Blood samples were collected from 1,454 subjects, ages 25–91. All blood samples were drawn between 8 and 11 a.m. For the measurement of biomarkers, blood was drawn into EDTA tubes. Plasma was obtained after centrifugation and was stored in 1-mL aliquots at −70°C until testing. The institutional review board at University of Texas Medical Branch approved the study protocol, and informed consent was obtained from all participants.

## Measurement of anti-HSV-1 and anti-CMV antibodies

Antiviral antibody titers were determined by indirect immunofluorescence (IF) as previously described [Stowe et al., 2001a; Stowe et al., 2000; Tingate et al., 1997]. Commercially prepared substrate slides and control sera (Microgen Laboratories, La Marque, TX, and Bion Enterprises, Park Ridge, IL) were used for determining IgG antibody titers to HSV-1. Briefly, 30 ul of titrated sera were pipetted onto spot slides and incubated for 30 minutes at room temperature. After incubation, the spot slides were rinsed for 5 minutes in PBS. The secondary antibody used was anti-human IgG conjugated with fluorescein isothiocynate. Evans blue was used as a counterstain. After a second incubation (30 minutes at room temperature), the slides were washed, lightly blotted, and mounted with mounting medium. Antibody titers were determined by the highest dilution of serum still able to demonstrate IF-positive cells. Sera from HSV-1 positive and negative individuals were used as controls. All specimens were batched analyzed and read blind-coded, and the standard error of the assay was ±1 dilution.

CMV IgG antibody levels were determined using enzyme immunoassay (Biocheck, Foster City, CA) according to the manufacturer's instructions. Briefly, all samples and reagents were brought to room temperature. Test samples and controls were diluted 1:40 with diluent. Then, 100 μl of the controls and samples were pipetted into wells. After a 30 minute incubation period at 37°C, the standards and samples were aspirated from the wells and washed with buffer using an Embla 96/384 well microplate washer (Molecular Devices, Menlo Park, CA). One hundred microliters of enzyme conjugate was dispensed into each well followed by another incubation period. The wells then were washed with buffer, and 100 μl of 3, 3', 5, 5'-tetramethylbenzidine subsequently was added followed by a 15 minute incubation period. Afterward, 100 μl of stop solution (1N HCL) was added. Absorbance was read at 450 nm using a SpectraMax Plus plated reader (Molecular Devices). The intra- and inter-assay coefficient variation ranges of this kit are 4.8% to 5.2% and 6.2% to 8.4%, respectively.

## Statistics

HSV-1 and CMV were examined as both continuous (antibody levels) and categorical measures (percent seropositive), and "high" CMV where high denotes IgG antibody values in the top quartile and "high" HSV-1 where high denotes HSV-1 titers ≥1280 [Nazmi et al., 2010; Stowe et al., 2007; Stowe et al., 2010; Tingate et al., 1997]. The extent to which mean CMV titer levels, percent of respondents having high CMV antibodies, and percent of respondents being CMV seropositive varied by age groups (< 45, 45–64, and 65+) was first assessed. These analyses were duplicated for HSV-1. Second, to determine more specifically how levels of CMV and HSV antibodies varied by age group, mean antibody levels by 10 year age groupings were examined, with a test for a linear trend. All statistical analysis of mean HSV-1 and CMV antibodies were performed using ANOVA, and the Chi-square test was utilized to examine patterns by age group for percent of respondents who were CMV or HSV seropositive and those who were coded as being high for CMV or HSV IgG antibodies. Finally, logistic regression was performed to examine the association between

having high CMV and having high HSV while adjusting for body mass index (BMI), gender, and socioeconomic status (SES).

## RESULTS

The mean age of the 1454 subjects was 48.9 years. Over half (58.4%) were female. Eighty-four percent of all subjects were CMV seropositive, and greater than 95% of all subjects were infected with HSV-1. As shown in Table 1, a higher percentage of respondents over the age of 65 were CMV seropositive (91.2% versus 77.5% for those under age 45, $p < .001$), though the age differences for being HSV-1 seropositive were not significant. In addition, respondents who were 45–64 had a significantly higher mean CMV titer levels than younger respondents. HSV-1 mean titer levels also varied by age. Respondents younger than 45 had significantly lower mean HSV-1 titers than those over the age of 45 ($p<.05$). Finally, a higher percentage of older respondents in this survey had high levels of both CMV and HSV-1 titers ($p<.001$).

To investigate age differences in CMV and HSV-1 antibody levels, additional ANOVAs for mean titer levels by 10-year age groups were conducted. Figure 1 shows the graph of the means for CMV and HSV-1 antibody levels. These data indicate that older respondents have higher levels of both HSV-1 and CMV titers ($p < .001$ for linear trend). Between the ages of 21–50, there was a steep rise in anti-CMV antibodies. Thereafter, levels of anti-CMV antibodies remained stable but elevated. Anti-HSV-1 antibodies exhibited a slower rise with age with titers peaking by age 70.

To show an association between CMV seropositivity and HSV-1 antibody levels, the 1454 subjects were divided into those previously infected with CMV and those who were seronegative. Mean antibody titers to HSV-1 were significantly higher in those respondents who were CMV seropositive, and the percentage of subjects with high HSV-1 titers was significantly higher in CMV seropositive subjects as compared to those who were seronegative ($p<0.05$; data not shown).

In the next set of analyses, subjects were subdivided into three age groups to assess differences in antibody levels to HSV-1 and CMV infection (i.e., seropositivity) by age. For each age group, CMV seropositive subjects consistently had higher HSV-1 titers than those who were seronegative (Figure 2). However, this difference was only significant among those respondents who were <45 years of age ($p<.05$). When comparing CMV seropositive subjects across age groups, a higher percentage of respondents who were older had elevated HSV-1 titers as compared to younger respondents ($p<.001$); there were no age differences for those who were CMV seronegative.

Finally, it was determined whether there was an association between high CMV antibody levels and high HSV-1 titers. There was a significant increase in the percentage of subjects with high HSV-1 titers in those individuals with high CMV antibodies who were <45 years of age as compared to those with low CMV antibody levels (Figure 3); no significant differences were found in the 45–64 and 65+ age groups. When comparing subjects with low CMV antibodies across age groups, a higher percentage of respondents who were older had elevated HSV-1 titers as compared to younger respondents ($p<.001$); there were no age differences for those who had high CMV antibodies. Notably, these associations remained significant after accounting for BMI, gender, and SES (data not shown).

## DISCUSSION

Exposure to CMV may have important health consequences since recent work suggests that CMV may reduce the capacity of the immune system to respond to antigenic challenge

[Khan et al., 2004; Pawelec et al., 2004]. Our goal was to extend these earlier findings by testing the association between CMV and HSV-1 antibodies in a large sample number that included a broad range of ages. In the present study, increases in CMV and HSV-1 antibodies were found in older adults as well as an increase in the percentage of subjects with elevated herpesvirus antibodies indicative of subclinical reactivation. These results are consistent with prior studies of latent herpesvirus reactivation in the elderly [Glaser et al., 1985; Musiani et al., 1988; Weymouth et al., 1990; Stowe et al., 2007] which can be attributed to age-related down-regulation of cellular immunity [Miller, 1991; Effros, 2000; Effros et al., 2003].

When subjects were grouped according to CMV serostatus, it was found that CMV seropositive individuals had both greater mean HSV-1 antibody levels as well as a higher percentage of individuals with high HSV-1 titers. Evaluation among different age groups showed that the association between CMV seropositivity with HSV-1 antibody levels was evident early in life as significant differences in HSV-1 titers were found in adults between the ages of 25–44 but not after age 44. In addition, there was a significant increase in the percentage of older subjects with high HSV-1 antibodies in CMV seropositive subjects but not CMV seronegative subjects. These data are consistent with previous studies showing that CMV infection has a profound impact on the immune system very early in life [Kaye et al., 2008; Miles et al., 2008; Chidrawar et al., 2009].

When assessing CMV antibody levels as a determinant of high HSV-1 antibodies, it was again found that there was a significant correlation between high levels of both CMV and HSV-1 antibodies in individuals <45 years of age. Between age groups, there were no significant changes in high HSV-1 antibody levels in individuals with high CMV antibodies. Notably, there was a significant increase in HSV-1 antibodies in older CMV seropositive adults that did not have high CMV antibody titers. This is in contrast with CMV seronegative individuals, in which no age-related increase in HSV-1 antibodies was observed. This is a noteworthy finding which suggests that the immune system of CMV negative older adults is able to moderate HSV-1 later in life, whereas the immune system of CMV-infected older adults eventually succumbs to the influence of CMV-induced immune dysregulation resulting in HSV-1 reactivation. Altogether, these data suggest that immunosenescence associated with CMV may occur early (<45 years of age) in life.

It is unknown whether the findings related to CMV are solely due to CMV-induced T cell dysregulation, or if other processes are involved. For instance, inflammation due to cellular stress (e.g., CMV reactivation, infection with another pathogen, injury, etc.) may activate the hypothalamic pituitary adrenal axis thereby resulting in HSV-1 replication [Turnbull and Rivier, 1995; Noisakran et al., 1998; Trzonkowski et al., 2003]. In addition, antigen-specific CD8+ T cells can produce TNF-α and IFN-γ [Komanduri et al., 2001; Almanzar et al., 2005], and these cytokines are necessary for the development of CMV-permissive macrophages [Docke et al., 1994]. Thus, inflammatory cytokines produced by activated T cells may facilitate additional latent CMV reactivation in macrophages [Soderberg-Naucler, 2006].

These results support and extend prior studies [Khan et al., 2004; Pawelec et al., 2004] and demonstrate immune dysregulation in CMV infected individuals. Notably, elevated levels of CMV antibodies have been associated with increased IL-6 and TNF-α levels in older adults [Trzonkowski et al., 2003; Wikby et al., 2005; Wikby et al., 2006] that may have important health consequences. Higher CMV antibody titers are also associated with increased CRP levels [Bennett et al., 2011]. CMV, and more recently EBV, have been implicated in the development of coronary artery disease [Kendall et al., 1992; Waldman et al., 2008]. Strandberg and coworkers [Strandberg et al., 2003] found that HSV-1 and CMV were

associated with cognitive impairment in elderly adults with cardiovascular disease. A subsequent study identified CMV as a predictor of cognitive impairment even after controlling for numerous covariates including age, education, and health conditions [Aiello et al., 2006]. In perhaps the most striking study, Wikby *et al.* [2005] found that the immune risk phenotype, characterized in part by co-infection with multiple herpesviruses, was significantly associated with cognitive impairment; the individuals with cognitive impairment were all deceased at follow-up which was attributed to allostatic overload due in part to multiple herpesvirus infections. As such, CMV (and other herpesviruses) may trigger immune dysregulation involved in a number of age-related diseases that may provide a pathway through which infected subjects may experience poorer health outcomes. Future studies are needed to elucidate the role of herpesviruses in healthy aging.

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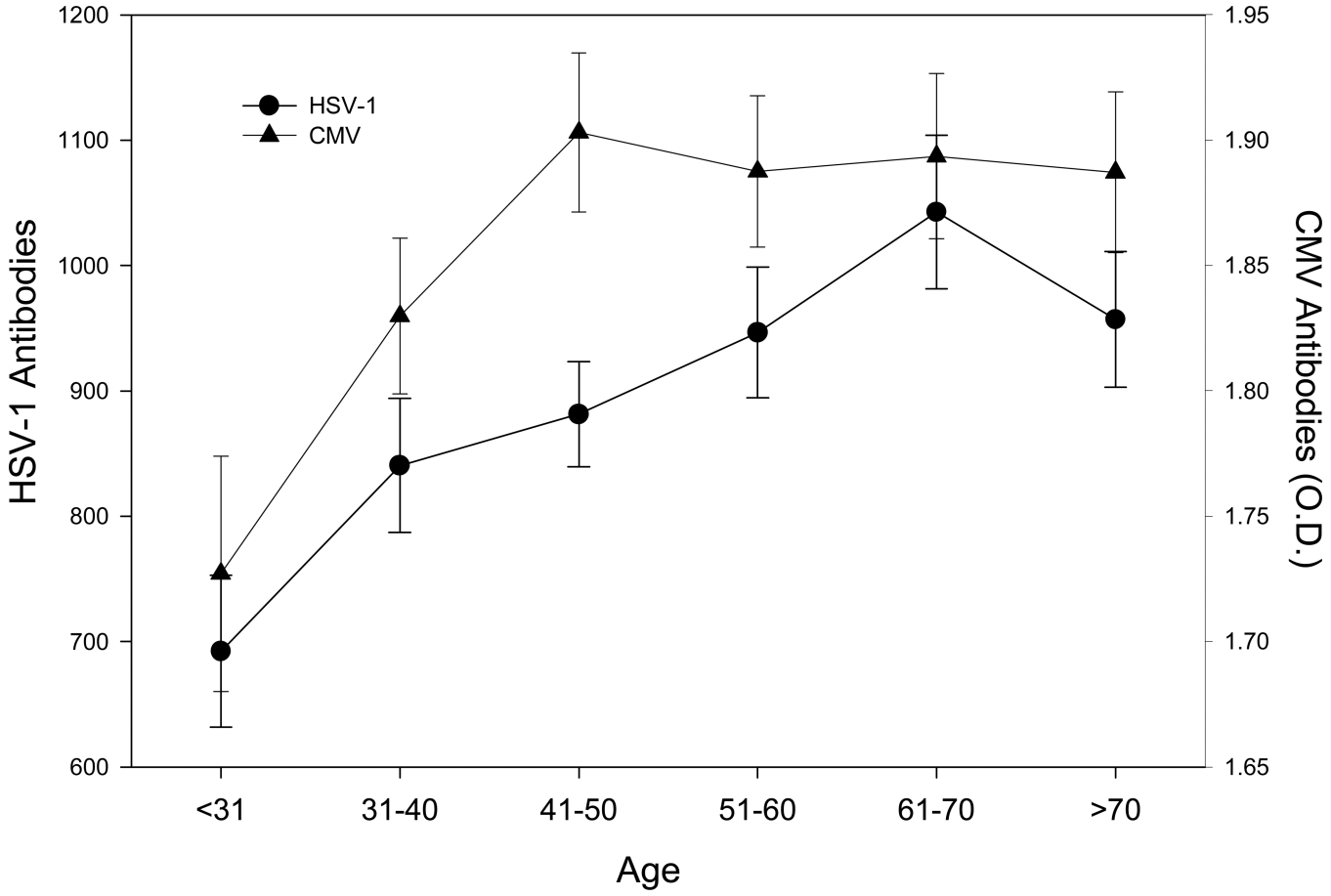


**Figure 1.**
Levels of anti-HSV-1 and anti-CMV antibodies (mean ± *SE*) by decade (*n* =1454).

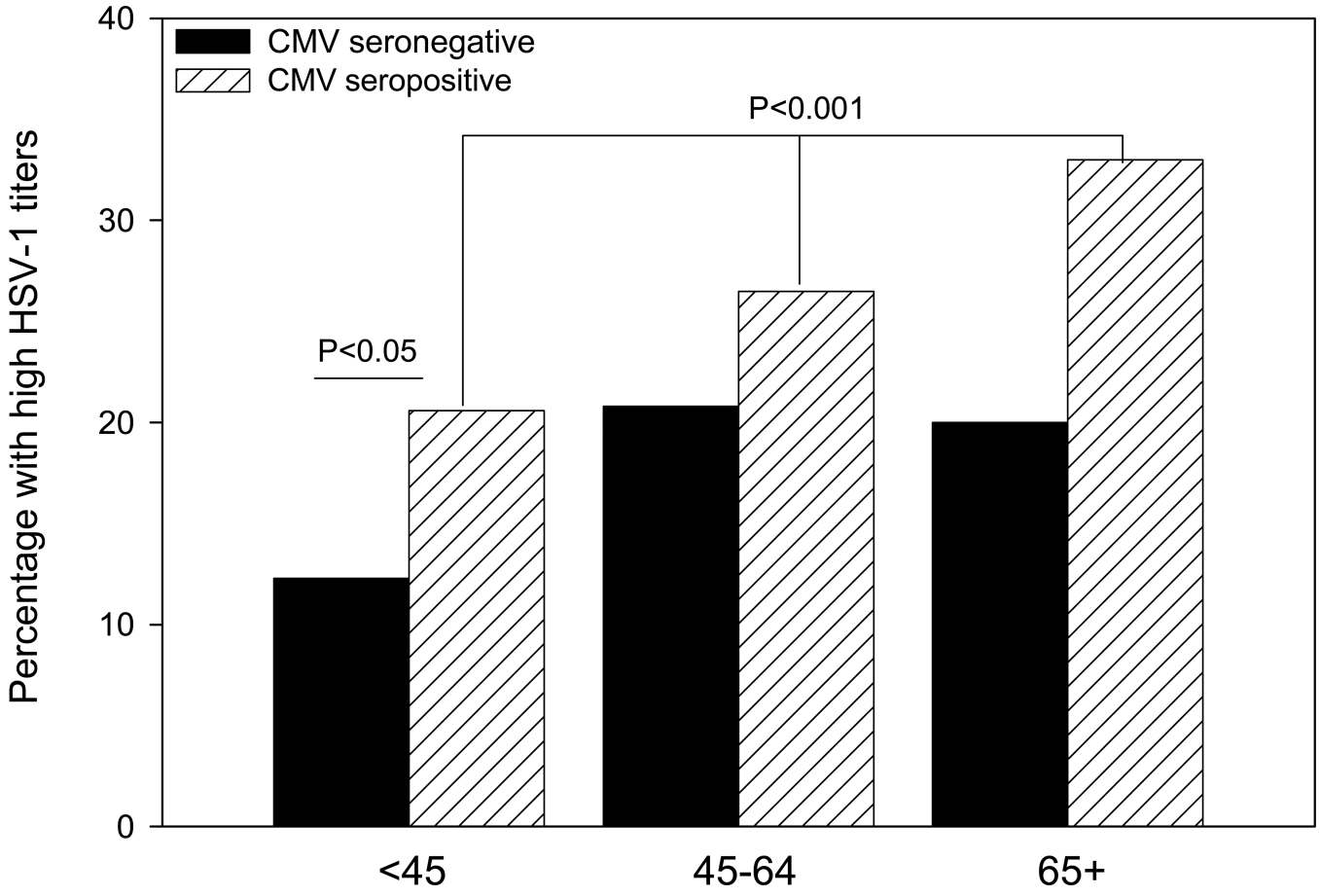


**Figure 2.**
Relationships between CMV serostatus and percentage of subjects with high HSV-1 titers within and between age groups.

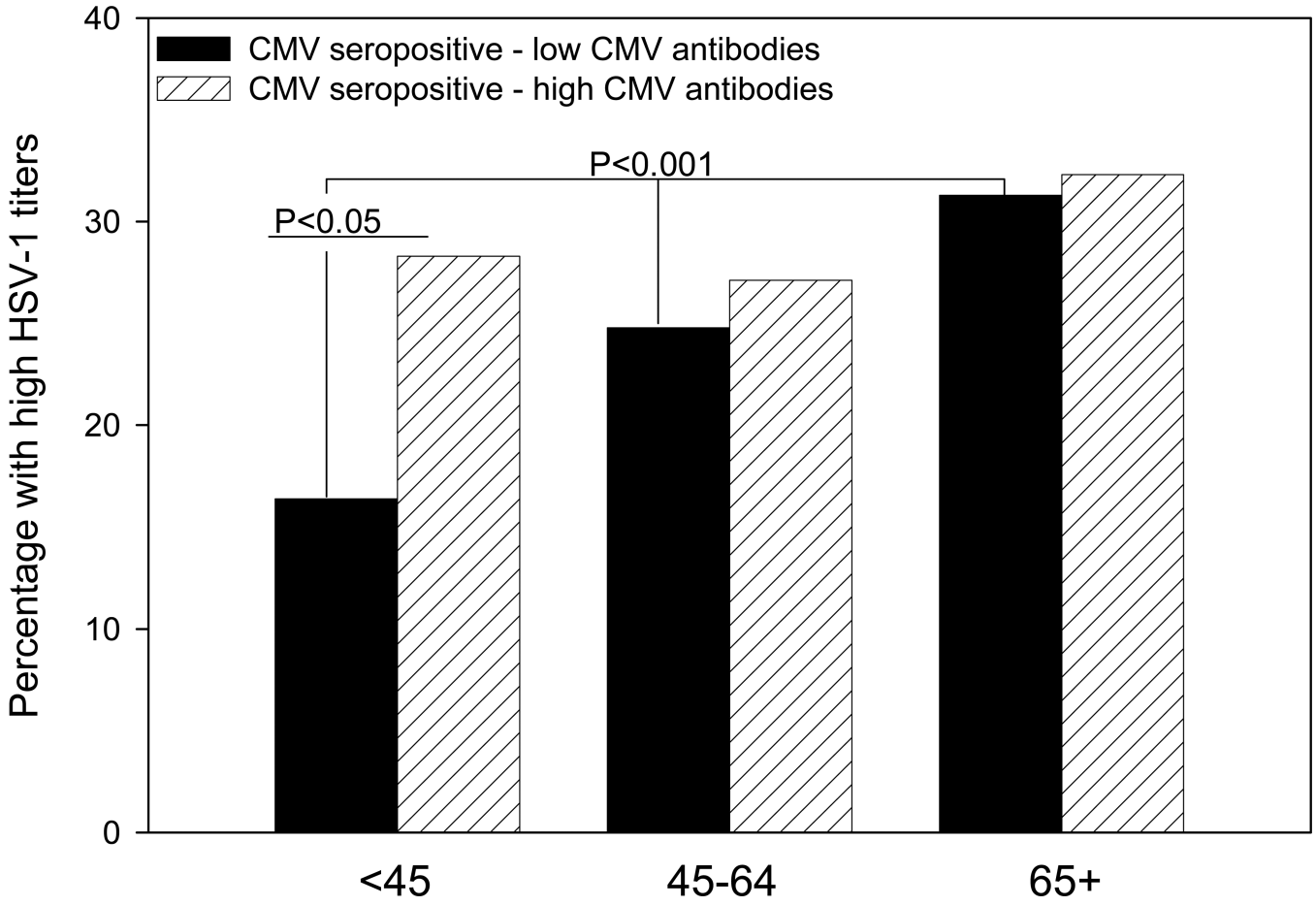


**Figure 3.**
Relationships between high CMV antibody levels and percentage of subjects with high HSV-1 titers within and between age groups.

## Table 1

HSV-1 and CMV Serostatus and Antibody Levels by Age from the Texas City Stress and Health Study (n = 1454)

| | <45 | 45–64 | 65+ | |
|---|---|---|---|---|
| | | | | $Chi^2$ P value |
| *n* | 406 | 580 | 468 | |
| % CMV seropositive | 77.5 | 82.6 | 91.2 | 0.001 |
| Mean CMV titer[a] | 1.82[d] | 1.91 | 1.87 | |
| % with high CMV antibodies[b] | 19.4 | 30.0 | 26.4 | 0.001 |
| % HSV-1 seropositive | 92.0 | 98.0 | 98.0 | 0.747 |
| Mean HSV-1 titer | 795.4[e] | 950.1 | 1034.9 | |
| % with high HSV-1 titers[c] | 18.8 | 25.5 | 31.5 | 0.001 |

Note: Age-group differences assessed by ANOVA for mean CMV and HSV-1 antibodies and through chi-square for percentage CMV and HSV-1 seropositive and for percentage with high CMV and HSV-1 antibodies.

[a] Measured in optical density unit

[b] High CMV defined as IgG values in the top quartile for this sample

[c] High HSV-1 defined as HSV-1 antibodies ≥1280

[d] Significantly different ($p<0.05$) from 45–64

[e] Significantly different ($p<0.05$) from 45–64 and 65+