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Are the Healthy Vulnerable? Cytomegalovirus Seropositivity in Healthy Adults Is Associated With Accelerated Epigenetic Age and Immune Dysregulation

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Background. Evaluating age as a risk factor for susceptibility to infectious diseases, particularly coronavirus disease 2019 (COVID-19), is critical. Cytomegalovirus (CMV) serologic prevalence increases with age and associates with inflammatory-mediated diseases in the elderly. However, little is known regarding the subclinical impact of CMV and risk it poses to healthy older adults. Prior to the COVID-19 pandemic we conducted a study to determine the association of CMV to biologic age and immune dysregulation.

Methods. Community-dwelling, healthy adults older than 60 years were evaluated using DNA methylation assays to define epigenetic age (EpiAge) and T-cell immunophenotyping to assess immune dysregulation.

Results. All subjects were healthy and asymptomatic. Those CMV seropositive had more lymphocytes, CD8 T cells, $CD28^-$ T cells, decreased CD4:CD8 cell ratios, and had higher average EpiAge (65.34 years) than those CMV seronegative (59.53 years). Decreased percent CD4 ($P = .003$) and numbers of CD4 T cells ($P = .0199$) correlated with increased EpiAge.

Conclusions. Our novel findings distinguish altered immunity in the elderly based on CMV status. Chronic CMV infection in healthy, older adults is associated with indicators of immune dysregulation, both of which correlate to differences in EpiAge.

Keywords. biologic age; COVID-19; cytomegalovirus; epigenetic age; immune dysregulation; seropositivity.

Age is a major risk factor for increased susceptibility to infectious diseases and decreased vaccine efficacy [1, 2]. Immune dysregulation and chronic subclinical inflammation contribute substantially to this risk [3]. Notably, dysregulation of the immune response occurs with viral infections, and was recently also described in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which disproportionately affects the elderly [4]. Cytomegalovirus (CMV), an almost ubiquitous human herpesvirus, is strongly associated with chronic subclinical inflammation, age-associated comorbidities, and immunosenescence [5, 6]. Large studies in octa- and nonagenarians have linked a dysregulated immune profile to an increased 2-year mortality [7]. On the one hand, a low CD4:CD8 T-cell ratio (<1) in CMV-positive individuals helped define the immune risk phenotype, which has been associated with increased risk of all-cause mortality [8]. On the other hand, a high CD4:CD8 T-cell ratio (>5) in CMV-positive individuals has been linked to impaired physical functioning in the elderly [9]. Herpesviruses, and in particular CMV, have an ability to initiate and maintain chronic immune activation and dysregulation, characterized by a progressive oligoclonal expansion of $CD4^+$ and $CD8^+$ effector memory T cells (Tem) [10]. In acute viral infections, once the infection clears, these T-cell populations normally contract [10]. In contrast, the Tem cell pool following CMV infection increases, creating Tem inflation and a concomitant peripheral naive T-cell decline, characterizing a state of chronic immune dysregulation [11, 12].

Chronic viral infections can accelerate biological aging, as determined by epigenetic changes. This was first shown in those with human immunodeficiency virus (HIV), where an average age acceleration of 5.2 years was described [13]. Epigenetic age (EpiAge) was determined by measuring methylation profiles of sites in the human genome whose methylation state correlates with age [14]. The EpiAge can also be used to calculate epigenetic age acceleration, which is a measurement of the difference between the observed EpiAge and a predicted EpiAge, from a linearized control model [13]. This tool may be useful as a clinical marker of disease progression. Additionally, hyper methylated *ELOVL2* CpG islands have been shown to be a marker of aging and cell replication in peripheral blood [15]. DNA methylation of 2 CpG sites proximal to the gene exhibit high correlation with age [15]. In the elderly and those with HIV, CMV seroprevalence is exceedingly high [16, 17]. It remains unclear

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to what extent latent CMV infection, rather than HIV, accounts for the epigenetic age acceleration, and if it predisposes to comorbidities frequently found in aging.

CMV-specific T cells account for a significant portion of the Tem pool in the elderly, with estimates ranging from 10% to 45% of the total $CD8^+$ T and 10% of the total $CD4^+$ T-cell populations [18, 19]. These CMV-specific T cells have an altered immune phenotype, are characterized by decreased cell surface expression of CD28, and by increased expression of KLRG-1 and CD57. These markers identify cell populations that have undergone repeated antigen stimulation [20, 21]. Furthermore, CMV-specific $CD8^+$ T cells reexpress CD45RA, a marker usually seen on naive T cells [22]. These cells have been termed effector memory reexpressing CD45RA T cells (TEMRA), and are found in increasing numbers with age in the bone marrow and peripheral blood [23]. The immunopathology contributing to the dysregulated T-cell phenotype is unknown.

CMV-associated T-cell alterations may have important consequences for immunosenescence, rates of aging, and age-associated comorbidities. Despite the challenge of identifying CMV uninfected seniors without comorbidities, we undertook a comparative study of healthy, older CMV-positive and CMV-negative adults to identify phenotypic and epigenetic changes associated with aging.

METHODS

Adults attending at 2 McGill University Health Centre (MUHC)-affiliated family medicine sites for their annual health care evaluations were screened for enrollment. MUHC Research Institute Review Board study approval was obtained. Based on predetermined inclusion and exclusion criteria, individuals were enrolled if they met the following inclusion criteria: age 60 years or older, in good health, and willing to provide a single 40-mL blood sample. Individuals were excluded if they were symptomatic, had an active or known chronic infection, a history of malignancy, autoimmune disease, diabetes, cardiovascular disease, or used immune modulators at any time. Informed consent was obtained from all study participants. All individuals were examined by a physician. The healthy status was defined using a strict version of the SENIEUR protocol criteria that also excluded those with any previous cardiac events such as myocardial infarction or stroke [24]. The SENIEUR protocol was used for this study as it was previously created to specifically limit the influence of disease and/or medication and to standardize admission criteria for immune studies in geriatric populations. Demographic, lifestyle, and clinical data were recorded.

White cell count and differential were performed on fresh blood as a standard of care. Absolute CD4 and CD8 counts were determined using the proportion of absolute lymphocytes expressing each of these surface markers. Flow cytometric analyses of lymphocyte subsets and serological assays for CMV were performed using serum and peripheral blood mononuclear cell (PBMC) samples. PBMC samples were divided in 3 aliquots, frozen in 10% dimethyl sulfoxide, and stored in liquid nitrogen immediately after being drawn. Plasma was stored at −80°C for CMV serology that was determined using a qualitative anti-CMV immunoglobulin G enzyme-linked immunosorbent assay (IgG ELISA; ABCAM), with duplicates run for each sample.

Targeted DNA Methylation Assays

A Pearson correlation between states of methylation of cytosine/guanine sites across the genome in blood cells from publicly available Illumina450K arrays and age (GSE61496) revealed that 2 sites residing proximal to the *ELOVL2* gene (cg16867657 and cg21572722) exhibit a strong Pearson product-moment correlation coefficient ($r = 0.934$, $P < .0001$) and ($r = 0.81004$, $P < .0001$). We developed a targeted DNA methylation assay to this region. A weighted EpiAge value was calculated for the 13 CGs in this region using a linear regression model based on measuring the state of DNA methylation of *ELOVL2*. This region describes the highest correlation with aging amongst 450 000 CpG sites included in Illumina methylation arrays and its methylation state has been correlated with age [25].

One million PBMC cells were placed in DNA stabilization buffer (sodium dodecyl sulfate 0.5%, EDTA 20mM, TritonX100 1%, Tris-HCl 20 mM pH8.0). Bisulfate conversion was performed using EZ-96 DNA Methylation MagPrep (D5041; Zymo Research), which was followed by 2 rounds of polymerase chain reaction (PCR); the first round targeted the *ELOVL2* and the second PCR introduced barcode sequences for multiplex amplification using the following primers: ELOVL2-Fw, YGGGYGGYGATTTGTAGGTTTAGT; ELOVL2-Rv, CCCTACACRATACTACTTCTCCCC. The pooled library was purified and sequenced on Illumina MiSeq, fast Q files were aligned with reference genome using BisMark, and percentage of DNA methylation at each of the 13 CG positions was computed. EpiAge was calculated with a linear regression equation predicting age in the control group as a function of the weighted methylation levels of all CG sites.

Lymphocyte Phenotyping

Batches of cryopreserved PBMCs were thawed and washed twice in phosphate-buffered saline. One million cells were stained with the following antibodies: BV650-CD3, BUV737-CD4, APC R700-CD8, BUV395-CD38, BV785-HLA-DR (Biolegend), and Indo-1-Live (Invitrogen).

All surface staining was done at room temperature for 30 minutes in the presence of human Fc block (BD Biosciences). All samples were analyzed on a BD LSR Fortessa X-20 (BD Biosciences). Fluorescence data from at least 50 000 lymphocytes were acquired. Analysis of data was performed using FlowJo version 10.

Statistical Analysis

Strict exclusion criteria, based on the SENIEUR protocol, were used during study enrollment. Potentially confounding mild ailments were allowed. To be certain that our findings were due to CMV infection alone, a variety of statistical tests were used to both ensure there were no significant differences in these aliments between CMV-positive and CMV-negative groups, as well as to control for these variables during significance testing.

A 2-sided Fisher exact test was used to determine significant differences in sex and smoking status between CMV-positive and CMV-negative groups. An unpaired 2-tailed *t* test was used to determine significant differences in body mass index (BMI) and age between the CMV-negative and CMV-positive populations.

A multilinear regression model was generated to control for sex, age, weight, height, BMI, and smoking status. The least squares method was used via Prism 8, assuming a Gaussian distribution of residuals. This model was used to test for significant differences between CMV-negative and CMV-positive populations. The following equation defines the model used to investigate each dependent variable investigated in this study:

$$\text{Dependent (eg, CD4 : CD8 ratio)} : Y = \beta 0 + \beta 1 \times \text{CMV} + \beta 2 \times \text{sex} + \beta 3 \times \text{age} + \beta 4 \times \text{smoking} + \beta 5 \times \text{BMI} + \text{random scatter}$$

where female = 0, male = 1; CMV negative = 0, CMV positive = 1; smoking no = 0, yes = 1; and β is the intercept parameter. Smoking status and BMI were unavailable for 5 individuals.

This model was applied to all T-cell phenotyping, replacing the dependent variable for each analysis. The following parameters were analyzed using this model: CD4:CD8 T-cell ratio, lymphocyte count, absolute CD4, percent CD4, absolute CD8, percent CD8, percent $CD4^+CD28^-$, and percent $CD8^+CD28^-$.

The same multilinear regression model was used to determine the impact of CMV status on EpiAge by imputing EpiAge as the dependent variable. Age acceleration due to CMV was calculated as previously described, where epigenetic age acceleration was defined as the difference between observed EpiAge and predicted EpiAge [13]. To generate the predicted EpiAge, a simple linear regression was created using the CMV-negative control population (Y = 0.5527*X + 20.43), where Y is chronological age and X is EpiAge. This equation was used to calculate predicted EpiAges for all study individuals. The difference between the predicted and actual EpiAge was calculated to produce epigenetic age acceleration values for all individuals.

A separate multilinear regression model was generated to determine the impact of EpiAge on CD4:CD8 T-cell ratio, lymphocyte count, absolute CD4, percent CD4, absolute CD8, percent CD8, percent $CD4^+CD28^-$, and percent $CD8^+CD28^-$. The above equation was used, substituting CMV with EpiAge (β1*EpiAge).

The Bonferroni correction was used for dependent variables that were tested in both multilinear regression models. *P* values were considered significant at < .025 for the following dependent variables: CD4:CD8 T-cell ratio, absolute CD4, percent CD4, absolute CD8, percent CD8, percent $CD4^+CD28^-$, and percent $CD8^+CD28^-$. All other *P* values were considered significant at < .05. All significance tests were carried out using GraphPad Prism 8.

RESULTS

A total of 520 individuals, sequentially presenting for annual clinical assessments, were evaluated as potential study participants. Of these, 429 were deemed not eligible because of current or past illnesses that included malignancy, autoimmune disease, diabetes, cardiovascular disease, or use of immune modulators. Of the 91 persons that met the study enrollment criteria and signed informed consent, 2 refused phlebotomy and 2 although healthy at screening were found to have active infection at the time of the blood draw. These 4 were excluded from the study and the remaining 87 subjects enrolled and completed the study. These individuals had minor clinical, stable conditions (hypertension, hypothyroidism, osteoporosis, dyslipidemia, benign prostatic hypertrophy, dyslipidemia, dyspepsia, and depression). Ages ranged from 60 to 90 years, with 65 subjects CMV positive and 22 CMV negative. The 2 groups did not significantly differ in sex, age, BMI, and smoking status (Table 1).

CMV-Seropositive Individuals Had Higher Lymphocyte Counts

The complete blood counts carried out on each individual revealed no significant differences in red blood cell, hemoglobin, leukocyte, or neutrophil counts between the CMV-positive and CMV-negative groups. The CMV-positive group, however, had a significantly higher lymphocyte count (1.88×10^9 cells/mL) than the CMV-negative group (1.49×10^9 cells/mL) (*P* = .0112; Figure 1A).

Table 1. Study Population Characteristics

Characteristics	Human CMV Serum Antibody IgG Negative	Human CMV Serum Antibody IgG Positive	*P* Value
All participants, No.	22	65	
Male, No. (%)	13 (59.1)	32 (49.2)	.46
Female, No. (%)	9 (40.9)	34 (51.5)	
Covariables			
Age, y (Mean + SD)	72.7 (8.2)	70.3 (8.9)	.23
BMI (Mean + SD)	26.6 (4.8)	26.4 (4.1)	.85
Smoking status, No. (%)	0 (0)	5 (6.8)	.32

Abbreviations: BMI, body mass index; CMV, cytomegalovirus; IgG, immunoglobulin G.

A 2-sided Fisher exact test was used to determine significant differences in sex and smoking status between CMV positive (n = 65) and CMV negative (n = 22). An unpaired 2-tailed *t* test was used to determine significant differences in BMI and age between CMV positive and CMV negative populations. *P* values below .5 were considered significant.

CMV-Positive individuals Had a Decreased CD4:CD8 T-Cell Ratio

CMV infection was associated with a decrease in the CD4:CD8 T-cell ratio (2.85 vs 4.27, $P = .0084$; Figure 1B). The MUHC established normal CD4:CD8 ratios are 1.8–3.4. We defined ratios of < 1 indicative of relevant dysregulation based on the OCTA and NONA studies [26]. To assess the degree of T-cell phenotypic dysregulation, the CMV groups were stratified on very low < 1.0, extended normal 1.1–4.9, and very high > 5.0 ratios, based on the OCTA and NONA studies (ratio < 1) and BELFRAIL study (ratio > 5) indicating negative clinical outcomes [9, 26]. The CMV-seronegative population had a lower proportion of individuals with a very low T-cell ratio < 1 (4.6%) compared to the seropositive population (16.9%).

Differences in T-Cell Subsets Between CMV-Positive and CMV-Negative Populations

The percentages of CD4 and CD8 T cells were calculated from live $CD3^+$ lymphocytes. There were no differences in CD4 percentage and absolute numbers between the 2 groups (Figure 2A). The CMV-positive population had a significantly higher percentage (22.78% vs 14.64%, $P = .0034$) and absolute number (0.44×10^9 cells/mL vs 0.22×10^9 cells/mL, $P = .0021$) of CD8 T cells as compared to the seronegative population (Figure 2B). In CMV seropositives, a significant increase in the percentage of $CD8^+CD28^-$ T cells (55.02% vs 22.95%, $P < .0001$) was noted (Figure 3). There was no significant difference in the percentage of $CD4^+CD28^-$ T cells between groups. There were no significant differences in the activation markers CD38 and HLA-DR between CMV-positive and CMV-negative populations.

Increased EpiAge Is Associated With CMV Infection

Samples at the time of EpiAge analysis were blinded to CMV serostatus. EpiAge was calculated for each study participant. Due to technical reasons, 14 samples failed to sequence. The CMV-positive group had a significantly higher EpiAge (65.34 years) than the CMV-negative control group (59.53 years) ($P = .0116$; Figure 4). We calculated age acceleration as previously described [13]. EpiAge (X) and chronological age (Y) in the CMV-negative group were plotted and the linear regression equation (Y = 0.5527*X + 20.43). We then computed the residual between the regression line and EpiAge in the CMV-positive group to calculate age acceleration. The result revealed a 5.1-year age acceleration in the CMV-positive group ($P = .0116$; Figure 5).

Increasing EpiAge Is Associated With Decreased Percentage and Absolute CD4 T Cells

A multilinear regression was carried out to determine the relationships between EpiAge and absolute and percent CD4, percent $CD4^+CD28^-$, absolute and percent CD8, percent $CD8^+CD28^-$, CD4:CD8 ratio, and immune risk phenotype. EpiAge positively correlated with decreased absolute ($P = .0199$) and percent CD4 T cells ($P = .0030$). Linear regressions were generated to visualize the relationship (Figure 6). There were no significant associations between EpiAge and CD8 T cells. CMV was the only variable that had significant effects on T-cell ratio and absolute and percent CD8.

DISCUSSION

The findings presented in this study reflect the older (60–90 years) population of Montreal, Canada. It is important

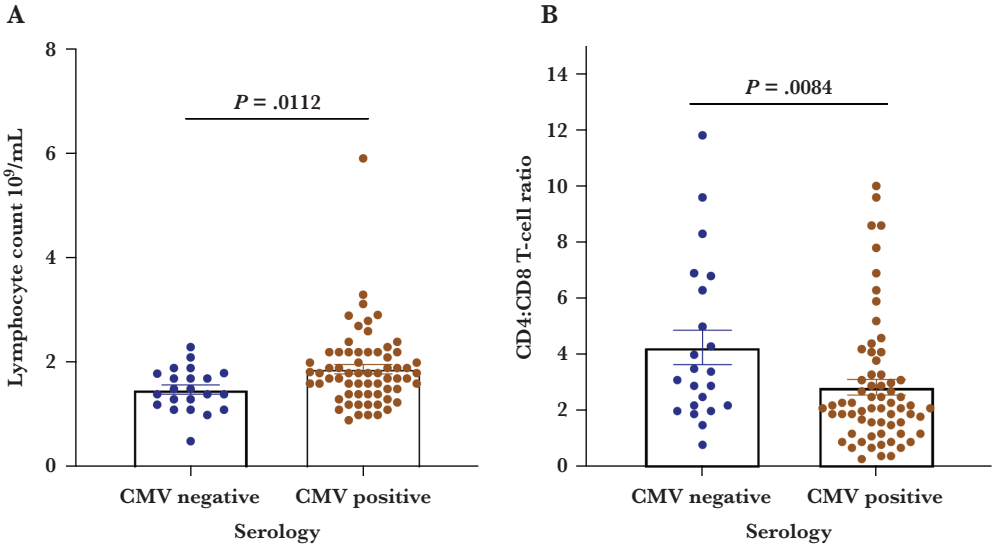

Figure 1. The CD4:CD8 T-cell ratio is decreased in the cytomegalovirus (CMV)-positive population. *A*, Lymphocyte counts were determined as a standard practice of care through the McGill University Health Centre. *B*, The CD4:CD8 T-cell ratio was determined for each individual using flow cytometry, gating on the $CD3^+$ population. The CMV-positive (n = 65) and CMV-negative (n = 22) populations were compared using multivariable linear regression models including age, sex, smoking status, and body mass index as covariables. A Bonferroni correction was applied and *P* values below .025 were considered significant. Each box represents the mean, the bars represent standard error and the dots represents each individual analyzed.

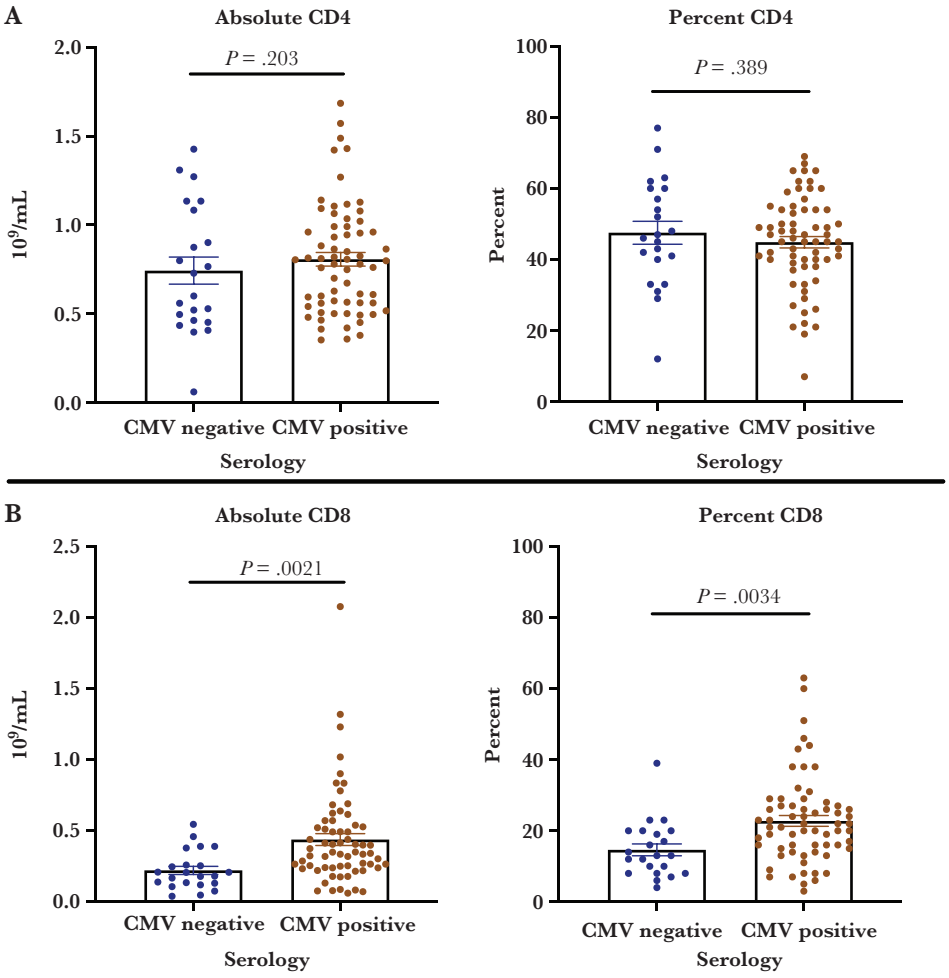

Figure 2. *A* and *B*, The absolute number and percentage of CD4 and CD8 T cells. The ratios, percentages, and absolute numbers of CD4 and CD8 T cells were determined for each participant using flow cytometry. The cytomegalovirus (CMV)-positive (n = 65) and CMV-negative (n = 22) populations were compared using multivariable linear regression models including age, sex, smoking status, and body mass index as covariables. A Bonferroni correction was applied and *P* values below .025 were considered significant. Each box represents the mean, the bars represent standard error and the dots represents each individual analyzed.

to note that the study was conducted prior to the coronavirus disease 2019 (COVID-19) pandemic. Furthermore, the recruitment of healthy seniors for this study was one of the major challenges, with the screening and recruitment process starting 4 years prior to completion of the study. A total of 520 adults older than 60 years were screened, with only 87 (16.7%) meeting the enrollment criteria. Of these, 25.3% were CMV negative. This highlights a common obstacle when researching CMV in the elderly. It is known that infection rates of the virus increase with age, and 74.4% of our study population was CMV positive, which matches other studies [27].

Our analysis outperforms those done in the BEFRAIL, OCTA, and NONA studies, as all of these studies explicitly mention that unknown disease states may have been a factor in their results [9, 28, 29]. Our strict screening allowed us to produce a more robust analysis. We included sex, age, BMI, and smoking status in our multivariate linear analysis, while eliminating diabetes, malignancy, infection, immune modulating drugs, cardiovascular disease, and autoimmune disorders at enrollment using entry criteria. We were still able to confirm the findings of the BEFRAIL, OCTA, and NONA studies, specifically an altered T-cell ratio and expansion of CD8 T cells.

We found that CMV infection was associated with an increased proportion of T-cell ratios of < 1. As previously shown by others, low T-cell ratios are largely due to an oligoclonal expansion of CMV-specific CD8 T cells [30]. Of importance, there was no decrease in the absolute number of CD4 T cells, but a significant increase in the absolute number of CD8 T cells. We have also shown an increase in the total lymphocyte count in the CMV-positive group. A similar finding was noted in human renal transplant recipients and in baboons [31, 32]. In CMV-positive individuals, an increase in lymphocytes is a function of age; however, we show it is also a function of CMV infection, as our CMV-positive group had a higher lymphocyte count than the CMV-negative group [33].

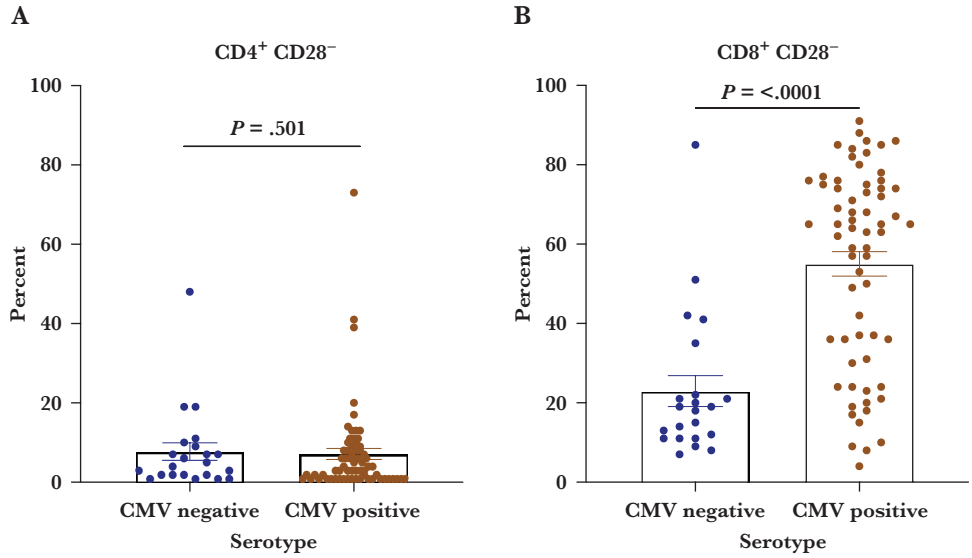

Figure 3. *A* and *B*, The CD8 T-cell phenotype is altered in cytomegalovirus (CMV)-infected participants. Peripheral blood mononuclear cells were isolated from CMV-negative and CMV-positive individuals. Flow cytometry was used to phenotype $CD3^+$ T cells from each individual participant, specifically analyzing CD4, CD8, and CD28. The proportions of CD28 for CMV-negative (n = 22) and CMV-positive (n = 65) individuals were calculated for $CD4^+$ and $CD8^+$ T cells. Multivariable linear regression models were used to test for significance including age, sex, smoking status, and body mass index as covariables. A Bonferroni correction was applied and *P* values below .025 were considered significant. Each box represents the mean, the bars represent standard error and the dots represents each individual analyzed.

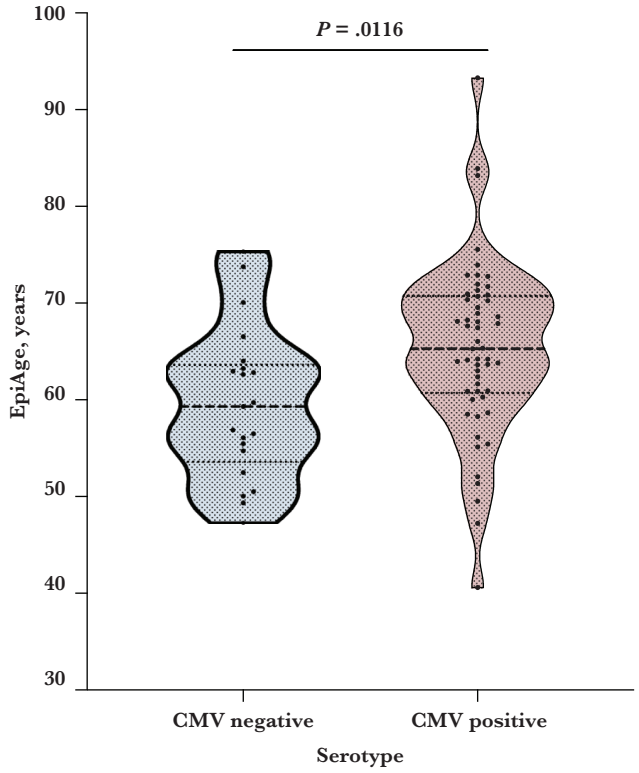

Figure 4. Epigenetic age (EpiAge) is accelerated in those with cytomegalovirus (CMV) infection. DNA was isolated from peripheral blood mononuclear cells of each sample and the methylation status near the *ELOVL2* gene was measured using bisulfite sequencing. An EpiAge was calculated for each participant, and the CMV-negative (n = 21) and CMV-positive (n = 54) groups were compared using a multilinear regression analysis including age, sex, smoking status, and body mass index as covariables. A violin plot was generated for each group, with the median and quartiles represented by dashed lines. *P* values below .05 were considered significant.

The effect of CMV infection on epigenetic aging was first described by Kananen et al [34], who found accelerated aging in CMV-positive young individuals between the ages of 20 and 30 years and also in the very elderly, aged 90 years and older. That study only had 6 CMV-negative controls in the aged older than 90 years group, and did not include anyone between the ages of 60 and 90 years. Our study expands on these findings by analyzing the missing age group, as well as making correlations between epigenetic age, a dysregulated T-cell phenotype, and the immune risk phenotype. As such, the findings have relevance regarding the use of EpiAge as a risk factor for many comorbidities and infectious diseases. The SARS CoV-2 pandemic has highlighted the importance of mortality risk stratification based on age, where the elderly account for 20% of those infected but 80% of deaths [35].

In healthy individuals older than 60 numerical years of age, our model indicated an age acceleration of 5.1 years solely due to CMV infection. Previous reports have associated CMV positivity with all-cause mortality and increased risk of cardiovascular disease in the general population, highlighting the serious impacts of chronic, untreated, subclinical infection [36, 37]. This effect is amplified in those living with HIV, where the vast majority of patients have CMV coinfections, leading us to dispute a 5.2-year average age acceleration finding attributed to HIV-1 alone, because the contribution of CMV was not considered in that study [13]. Ongoing antigenic stimulation from chronic viral infections, including CMV, results in premature T-cell exhaustion and accelerated telomere shortening [38]. In a prospective large cohort study, CMV seropositivity

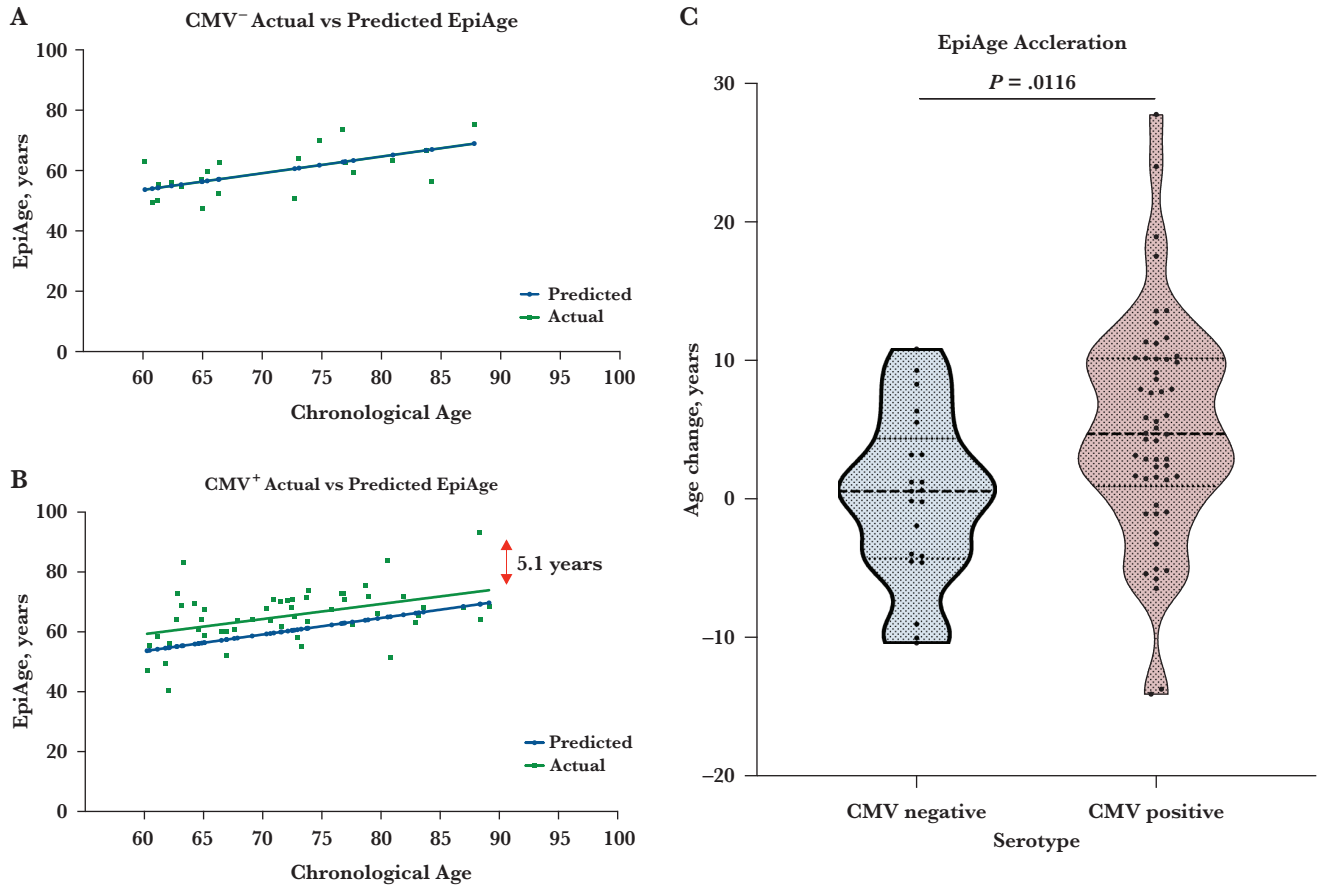

Figure 5. Cytomegalovirus (CMV)-associated age acceleration. The age acceleration due to CMV infection was determined by calculating the difference in the experimental epigenetic age (EpiAge) and predicted EpiAge. The predicted EpiAge was generated from a linear model based on the control CMV-negative group. *A* and *B*, The predicted and actual EpiAges were plotted against chronological age for CMV-negative and CMV-positive populations, and simple linear regressions were generated to visualize the relationship. *C*, Age acceleration was calculated for both CMV-negative (n = 21) and CMV-positive (n = 54) groups by calculating the difference between the predicted and actual EpiAges. The violin plot shows the frequency distribution of the data with a large dashed line at the median, small dashed line at the quartiles. Each dot represents an individual EpiAge. *P* values below .05 were considered significant.

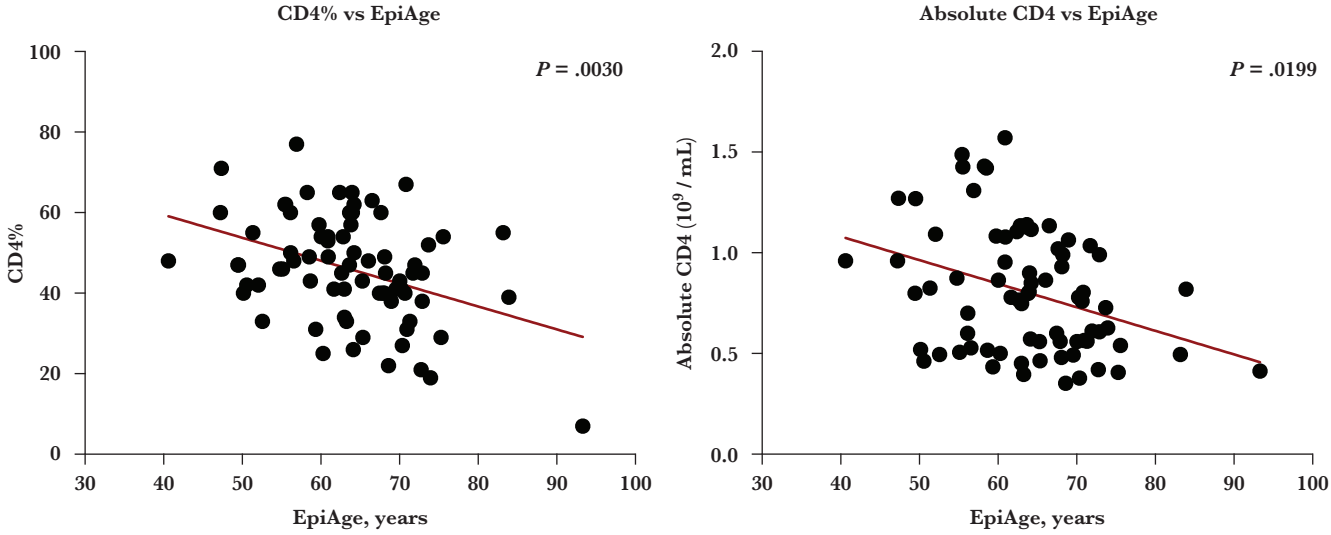

Figure 6. Increased epigenetic age (EpiAge) is associated with decreased CD4 T cells. Flow cytometry was used to phenotype T cells from each individual included in the EpiAge analysis. Percent and absolute number of CD4 T cells were plotted against EpiAge. Simple linear regressions were generated to visualize the relationship. Multilinear regression analyses including age, sex, smoking status, and body mass index as covariables were used to determine if EpiAge had a significant impact on CD4 T-cell percent and absolute count. A Bonferroni correction was applied and *P* values below .025 were considered significant.

was associated with shortened telomeres. Declines in telomere length occurred over a 3-year period and although the association was made with multiple herpes viruses, CMV was the main driver [39]. We have also previously demonstrated telomere shortening of lymphocytes in HIV-positive individuals receiving treatment having an immune risk phenotype, which is characterized by CMV seropositivity and low CD4:CD8 cell ratios [40].

Furthermore, CMV positivity is associated with a 2.5-fold increased risk of morality in AIDS patients, with CMV DNA load being a better predictor of mortality than $CD4^+$ count [41]. A CMV-negative/HIV-positive population is needed to truly elucidate the age-accelerating effects of HIV. Additionally, we showed that decreased percent and absolute number of CD4 T cells were associated to increases in EpiAge, identifying potential contributors to increased EpiAge. A cohort spanning additional chronological ages is needed to accurately investigate T-cell dysregulation and EpiAge.

A limitation of our study was that we did not phenotype CMV-specific T cells, rather we looked at T-cell subsets as a whole. Moving forward, it is important to determine if phenotypic changes occur in CMV-specific T cells, and if changes in CMV-specific T cells are correlated with chronological and EpiAge.

In conclusion, we identified new biomarkers indicating increased epigenetic age, accelerated aging, and immune dysregulation in healthy, asymptomatic, older adults with CMV. Because the COVID-19 pandemic has identified age as a major risk factor for infectious disease severity, our novel findings may have prognostic relevance. Additionally, we highlight the need to explore CMV vaccinations and prophylaxis with antiviral drugs, such as letermovir and valganciclovir, which have both been shown to reduce CMV infection and CD8 T-cell activation in the case of the later [42, 43]. Further studies are needed to determine these proposed interventions with respect to their potential clinical significance and effect on EpiAge.

Notes

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Author contributions. C. T. conceived and supervised the study, and recruited and enrolled study participants. C. T. and M. S. designed the experiments, and reviewed data and the analysis of results. C. P. collected and processed blood samples, did the immune assays, analyzed data, and prepared all figures. D. C. conducted the epigenetic studies. C. P., C. T., and M. S. drafted the original article. All authors edited the article, and read and approved the final version.

Disclaimer. The study sponsor had no role in the study design or the collection of data.

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Potential conflicts of interest. M. S. and D. C. have applied for patents on EpiAge. M. S. owns shares in HKG Epitherapeutics. All other authors report no conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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