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Presence of Asymptomatic CMV and EBV DNA in Blood of Persons with HIV Starting Antiretroviral Therapy are Associated with Non-AIDS Clinical Events

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Abstract

Background—Even with antiretroviral therapy (ART), persons with HIV (PWH) experience increased morbidity/mortality. Cytomegalovirus (CMV) and Epstein-Barr-Virus (EBV) co-infections likely exacerbate inflammatory-related diseases.

Objective—To determine if presence of detectable CMV or EBV DNA in peripheral blood mononuclear cells (PBMC) is associated with non-AIDS events among PWH receiving modern ART.

Design—We performed a case-control study of PWH starting ART and HIV-suppressed at year 1 and thereafter, 140 cases who experienced non-AIDS events and 305 matched controls. Events included myocardial infarction, stroke, malignancy, serious bacterial infection or death.

Methods—Blood samples were studied pre-ART, 1-year post-ART and pre-event. Controls had an event-free follow-up equal or greater than cases. CMV and EBV levels were measured in PBMC. Conditional logistic regression analysis assessed associations and adjusted for relevant covariates; Spearman's correlations compared CMV and EBV levels with other biomarkers.

Results—CMV was detected in PBMC of 25% of participants, EBV was detected in >90%. Higher EBV levels were associated with increased risk of events at all time points (odds ratio (OR) per one IQR = 1.5–1.7, all $p < 0.009$). At year 1, detectable CMV was associated with increased risk of events in most adjusted models (OR = 1.4–1.8, p -values ranging 0.03–0.17). Higher levels of CMV and EBV correlated with multiple inflammatory markers and lower CD4/CD8 ratio.

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Conclusions—In PWH starting ART, CMV and EBV in PBMC were associated with development of non-AIDS events. Clinical trials will be needed to understand causal mechanisms and ways to interrupt them.

Keywords

HIV; Inflammation; Non-AIDS event; CMV; EBV; Non-AIDS mortality; Viral suppression; CMV and EBV DNA

Introduction

Antiretroviral therapy (ART) improves health, prolongs survival and reduces HIV transmission [1]. Nevertheless, even when successfully treated, HIV infection remains associated with higher morbidity and mortality due to non-AIDS defining events, such as cardiovascular disease (CVD) and non-AIDS-defining malignancies [2]. This increased morbidity and mortality is also associated with inflammation and immune dysfunction, which persists in most people with HIV (PWH) despite modern ART [3]. Potential contributors to such inflammation include: direct effect of HIV expression, translocation of bacterial and fungal products from the gut into the systemic circulation, and chronic viral co-infections like cytomegalovirus (CMV) or Epstein-Barr Virus (EBV) [2, 4].

Nearly all PWH are co-infected with CMV and EBV, and most PWH experience intermittent bursts of CMV and EBV replication, even during ART [5]. Such replication is linked to repeated stimulation of the CD8⁺ T-cell population [6, 7], and presence of CMV DNA is associated with lower CD4/CD8 ratio during ART [6, 8, 9], and increased activation, cycling, and exhaustion of T-cells [10, 11]. Further, magnitude of humoral and cellular CMV-specific immune response among PWH is associated with cardiovascular disease and neurocognitive dysfunction [12–16]. During advanced stages of HIV infection, the immune system is often damaged to the point that it can no longer contain CMV replication, thus causing significant morbidity and mortality. Older studies among PWH not receiving modern ART found that CMV DNA in plasma consistently predicted mortality, mostly with AIDS associated events [17, 18]. However, the vast majority of PWH on modern ART are undetectable for CMV DNA in plasma, and yet continue to have residual inflammation and are at increased risk for adverse clinical outcomes [2]. Co-infection with EBV has also been associated with inflammation, end-organ diseases, and cancer [19, 20] among both PWH and HIV-uninfected persons, although less consistently than CMV.

Most previous studies evaluating the effect of CMV and EBV on end-organ diseases in the setting of HIV, have considered antibody responses and not viral replication [15, 16, 21, 22]. Thus, it is still unclear if identified end-organ diseases are directly associated with viral activity itself or are a consequence of the immune response towards CMV and EBV. The present study aimed to determine if presence of detectable CMV or EBV DNA in peripheral blood mononuclear cells (PBMC) is associated with non-AIDS events among PWH receiving modern ART.

Methods

Study Cohort and Samples

The study population includes treatment-naïve PWH who received initial treatment as part of a prior ACTG study (i.e. A388, A384, A5014, A5095, A5142, A5202) [23–29], see Supplementary Table 1 details. All 445 participants (140 cases who experienced non-AIDS events and 305 matched controls) were ART naïve at the time of enrollment. They all had a plasma HIV-1 RNA load of <400 copies/mL at week 48 after ART initiation and maintained a plasma HIV RNA load of < 400 copies/mL at subsequent visits (isolated values > 400 copies/mL were allowed if preceding and subsequent values were <400 copies/mL). All participants provided written informed consent, and institutional review board approval was obtained by each study site.

Cases and controls were originally identified as part of a prior case-control study [30]. Briefly, cases were defined as people who had a myocardial infarction (MI), stroke, non-AIDS-defining malignancy, serious bacterial infection (i.e. bacterial pneumonia/bronchitis, sepsis, or bacterial meningitis) or died from a non-accidental non-AIDS-related event after week 48 [30, 31]. For each case, 2 or 3 controls were identified who had an endpoint-free follow-up time equal or greater than that of the case and were matched on the basis of age (within 10 years; median 45 years), sex (84% male), baseline CD4⁺ T cell count (within 50 cells/mm³; median 219 cells/mm³), ART regimen at week 48 (whether it contained a protease inhibitor or abacavir), and parent study [30, 31]. In our current study, we included all cases and controls who had available PBMC specimens for testing. Controls without a corresponding case, and cases without at least one corresponding control were excluded.

Stored PBMC specimens were evaluated at the following 3 time points: before ART initiation (baseline); 48–64 weeks after starting ART (year 1); and the ACTG study visit immediately preceding the event for cases (median of 10.5 weeks [IQR: 6–19]) and a similar time point for corresponding controls (pre-event). PBMC samples were available for 232 participants at baseline (85 cases and 147 controls), 404 participants at year 1 (128 cases and 276 controls), and 347 participants pre-event (110 cases and 237 controls).

CMV and EBV Antibody (IgG) and DNA Levels

Anti-CMV and EBV IgG antibody levels were measured in blood plasma at year 1 using GenWay Biotech ELISA kits [22, 32, 33]. DNA was extracted from 5 million PBMCs at each time point using Qiagen QIAmp DNA Mini Kit. Total CMV and EBV DNA were quantified by droplet digital PCR (ddPCR) [22, 34, 35]. Copy numbers were calculated as the mean of replicate PCR measurements and normalized to one million cells, determined by the housekeeping gene Ribonuclease P/MRP Subunit P30 (RPP30) [36]. CMV and EBV IgG level < 1.2 IU/mL were considered as seronegative.

Inflammation Associated Soluble and Cellular Biomarkers

Cellular and soluble markers of inflammation, immune activation, and gut damage were generated as part of previous studies at baseline, year 1, and pre-event [30, 31]. These included plasma interleukin (IL)-6, Interferon gamma-induced protein (IP)-10, soluble

tumor necrosis factor receptors (sTNFR) -I, sTNFR-II, soluble cluster of differentiation (sCD)14, D-Dimer, Intestinal fatty-acid binding protein (I-FABP), Lipopolysaccharide binding protein (LBP), sCD163, soluble urokinase-type plasminogen activator receptor (suPAR), (1–3)- β -D-Glucan (BDG), and cellular %CD4⁺:CD28⁻CD57⁺, %CD8⁺:CD28⁻CD57⁺, %CD4⁺:CD38⁺HLADR⁺, %CD8⁺:CD38⁺HLADR⁺, %CD4⁺:PD1⁺, %CD8⁺:PD1⁺, and CD4/CD8 ratio.

Statistical Analysis

Non-normal data were log-transformed to the log₁₀ scale; values of zero were imputed to 0.1 prior to log-10 transformations. Descriptive summaries of continuous variables included the frequency, median, and interquartile range (IQR), while frequency and percentage were used for categorical variables. All statistical tests used a two-sided 5% significance level, without adjustment for multiple comparisons. We emphasized magnitudes of effect sizes, consistency of observed effects, and robustness to our adjusted models.

First, we assessed associations between CMV DNA, EBV DNA, antibody levels and previously generated soluble and cellular biomarkers using Spearman's correlations at concurrent time points. Only the controls were included in these analyses to provide a more generalizable estimate of the true association in the general population, as cases were enriched for Non-AIDS events.

Subsequently, we used conditional logistic regression analysis to determine if higher levels of EBV DNA or presence of CMV DNA were associated with increased risk of having subsequent non-AIDS events in a cross-sectional analysis at each time point. For this analysis, EBV DNA was analyzed continuously on the log₁₀ scale and was categorized by the pooled (combined cases and controls) IQR such that the levels of the biomarkers were grouped as <25th percentile, 25th-49th percentile, 50th-74th percentile, and 75th percentile. Due to the large proportion of undetectable by lab assay, CMV DNA was analyzed as a dichotomous variable: detectable (>0 copies/million) versus undetectable (0 copies/million). Models were individually adjusted for each covariate (i.e., HIV RNA level, CD4 cell count, Hepatitis B/C status, smoking status, history of injection drug use, diabetes, hypertension, use of any cardiac medications, waist-to-hip ratio, family history of MI); additional adjustments for previously analyzed biomarkers (as mentioned above) were also evaluated [30, 31]. Separate unadjusted conditional logistic regression models further examined four event subgroups: death, malignancy, cardiovascular (MI/stroke), and serious bacterial infections. For the death subgroup analysis of CMV DNA, due to the small number of deaths, we examined the association using the Cochran-Mantel-Haenszel test for stratified categorical data.

Sensitivity analysis was performed to assess the potential impact of false negatives in CMV and EBV DNA detection due to small low template input (in samples with low DNA concentration). Conditional logistic regression analysis was repeated excluding samples with the lowest template input (RPP30 levels <10th percentile).

Results

Cohort and Samples

All participants were seropositive for CMV and EBV IgG. Study participants who did and did not have non-AIDS events (i.e. cases and controls) had overall similar demographic characteristics at baseline, but differences were observed in the percentage with chronic Hepatitis B/C, history of hypertension, and smoking status (Table 1); models include adjustments for these characteristics. Among 140 cases, non-AIDS-defining events occurred at a median of 2.9 years (IQR: 1.8–4.7) after ART initiation. Among the cases, 15% were deaths, 29% were MI/strokes, 35% were malignancies, and 24% were bacterial infections; eight cases died from non-AIDS events (3 MI/strokes, 2 malignancies, 3 bacterial infections) and are included twice in these summaries.

Frequency and Levels of CMV and EBV DNA

CMV DNA was detected in 40% of PBMC samples at baseline, 24% at year 1 and 18% at pre-event. In contrast, >90% of all samples contained detectable EBV DNA at all time points (Figure 1 and Supplementary Table 2). At baseline, 36% of PBMC samples were detectable for both viruses (CMV and EBV), while 23% and 18% were detectable for both viruses at year 1 and pre-event, respectively.

CMV and EBV DNA Associations with Inflammation Among Controls

In analyzing associations between CMV DNA, EBV DNA, and antibody levels, there was a weak association between CMV DNA and EBV DNA at the pre-event time point ($r=0.18$, $p=0.005$), but no associations were seen at year 1 or baseline. At year 1, we observed a modest correlation between EBV DNA and EBV IgG antibody levels ($r=0.43$, $p<0.0001$), but not between CMV DNA and CMV IgG level. Figure 2 summarizes correlations between CMV and EBV DNA and inflammation associated biomarkers. Overall, levels of CMV DNA at baseline had modest positive correlations with all soluble inflammatory markers (r ranging between 0.18 and 0.35, $p<0.036$) and was negatively correlated with the CD4/CD8 ratio ($r=-0.39$, $p<0.0001$). These associations for CMV were not observed at year 1 or pre-event. On the other hand, EBV DNA level was only associated with IP-10 at baseline ($r=0.16$, $p=0.046$), which remained at year 1 ($r=0.18$, $p=0.003$). At year 1, levels of EBV DNA were also positively associated with sCD163, sTNFR-I, sTNFR-II, %CD8⁺:CD38⁺HLADR⁺, %CD4⁺:CD38⁺HLADR⁺, and %CD4⁺:PD1⁺ (r ranging between 0.14 and 0.22, $p<0.023$) and negatively associated with the CD4/CD8 ratio ($r=-0.17$, $p=0.005$). At pre-event, EBV DNA levels were also associated with IP-10 ($r=0.20$, $p=0.002$) and with the CD4/CD8 ratio ($r=-0.21$, $p=0.001$), and new associations were observed with IL-6 and D-dimers ($r=0.16$, $p<0.015$). Scatterplots of the raw data are included in Supplementary Figures 5A–F.

EBV DNA and CMV DNA as Predictors of Non-AIDS Events

Higher levels of EBV DNA were associated with increased risk of subsequent non-AIDS events at all time points (odds ratios per one IQR ranging 1.5–1.7, $p<0.009$). The strongest associations were observed at baseline. The effect persisted after adjusting for each of the clinical factors and inflammatory biomarkers (odds ratios per one IQR ranging 1.3 to 2.0)

(Figure 3 and Supplementary tables 3 A–F). Examining event sub-groups, higher levels of EBV DNA at baseline were associated with increased risk of MI/stroke (odds ratio per one IQR (95% CI) = 2.3 (1.1, 4.7)), and marginally with elevated risk of malignancy (odds ratio per one IQR (95% CI) = 2.4 (1.0, 6.0)). Although not significant, higher odds of death were observed for higher baseline EBV DNA levels (odds ratio per one IQR (95% CI) = 2.7 (0.5, 13.2)). At year 1 and pre-event, higher EBV DNA levels were associated with increased risk of death (year 1: odds ratio per one IQR (95% CI) = 3.1 (1.2, 8.1); pre-event: odds ratio per one IQR (95% CI) = 2.6 (1.0, 6.8), malignancy (year 1: odds ratio per one IQR (95% CI) = 2.1 (1.2, 3.6); pre-event: odds ratio per one IQR (95% CI) = 2.6 (1.4, 4.9)), and serious bacterial infection (year 1: odds ratio per one IQR (95% CI) = 1.9 (1.0, 3.4); pre-event: odds ratio per one IQR (95% CI) = 1.8 (0.9, 3.5)). Increased risk of MI/stroke was not seen for EBV DNA at year 1 or pre-event.

Similar to the EBV analysis, we observed a trend between having detectable CMV DNA in PBMC at year 1 and increased risk of non-AIDS events (odds ratio (95% CI) = 1.6 (1.0, 2.6), $p=0.07$); this effect persisted after adjustment for clinical factors (odds ratios ranging 1.4 to 1.7) (Figure 4 and Supplementary tables 4 A–C). The analyses were repeated after excluding participants with low template input (i.e. those in the lowest 10th percentile of RPP30). The results were unchanged at baseline and at the pre-event time points, but the results at year 1 showed stronger associations with significantly increased risk of non-AIDS events (odds ratio (95% CI) = 1.9 (1.1, 3.2), $p=0.017$) with the presence of CMV DNA. In event sub-group analyses, marginal associations were observed between CMV DNA and risk of malignancy and MI/Stroke at year 1 (malignancy: odds ratio (95% CI) = 1.8 (0.8, 4.1); MI/Stroke: odds ratio (95% CI) = 1.8 (0.7, 4.6)). The Cochran-Mantel-Haenszel test was used to evaluate the risk of death due to the small number of deaths and analytic convergence issues. There was an increased risk of death for those with detectable CMV DNA at baseline (odds ratio (95% CI) = 9.0 (1.9, 42.6)).

Discussion

Despite ART, HIV infection remains associated with higher morbidity and mortality, driven by increased inflammation [2], and chronic CMV and EBV co-infections may exacerbate this inflammation [5]. To better understand the impact of chronic EBV and CMV co-infection in PWH on modern ART, we examined associations between viral DNA in peripheral blood cells and non-AIDS events in a case-control study of 445 PWH who did and did not have non-AIDS events over time. The analysis evaluated associations with a composite non-AIDS endpoint, as chronic inflammation driven by CMV and EBV co-infections is a plausible mechanism underlying each of the non-AIDS events; event-specific analyses were also performed.

Overall, cellular CMV DNA was detected in 25% of all samples, while EBV DNA was detected in >90%. Higher levels of EBV DNA in blood cells were consistently associated with an increased risk of developing subsequent non-AIDS events, with the strongest associations between EBV DNA at baseline and subsequent non-AIDS events. Detectable CMV DNA at year 1 was weakly associated with increased risk of subsequent non-AIDS events in adjusted analysis, but this was not observed for CMV DNA in blood cells at other

time points. These results are consistent with a study that showed an approximately 50% higher risk of severe non-AIDS-defining events in PWH who were seropositive for CMV antibody [15], and other studies that described associations between CMV IgG or cellular immune response and non-AIDS events [22], in particular cardiovascular [12, 14] and neurological complications [16]. However, it is not clear what the correlations are between EBV and CMV replication and immune responses, especially as a previous study showed that higher CMV IgG levels were associated with lower CMV shedding in the setting of HIV [33]. Interestingly, in our study we did not see a correlation between CMV DNA in PBMC and CMV IgG levels at year 1, but we did see a positive correlation between EBV DNA and EBV IgG. This discordance could be due to the differences in EBV and CMV DNA detectability in blood cells or preferred sites of replication between viruses. Alternative explanations are that T cell immunity might reflect the burden of infection rather than humoral response, or that measurement of latent infection does not reflect the burden of active, lytic infection.

In people with intact immune system, CMV does not frequently replicate in blood but at other mucosal sites (e.g. salivary glands, genital tract, but, endothelial cells), while EBV commonly replicates in the circulating B cells [5]. Thus, the modest association observed between CMV DNA in PBMC and non-AIDS events and the lack of association between CMV and inflammation during ART (year 1 and pre-event) was likely because we did not collect and evaluate mucosal specimens. The presence of CMV DNA in blood cells (usually monocytes) may reflect low level viral replication but may also simply reflect latent CMV infection and not necessarily CMV replication. Also, the clinical significance of detectable CMV DNA in blood cells is unknown. Therefore, future studies of CMV replication and clinical outcomes should assess mucosal specimens, as well. In contrast, the frequency of EBV DNA in circulating blood cells (mostly B-cells [5]) was consistently associated with non-AIDS events before and after ART. The link between EBV levels and clinical outcomes merit further study to help determine if these are linked to each other through systemic inflammation or more directly.

We also explored associations between CMV and EBV replication and various inflammatory markers. As in earlier studies [4, 37], levels of CMV DNA in PBMC were positively correlated with several soluble inflammatory markers at baseline, but not at subsequent time points. EBV DNA levels in PBMC were correlated with a few biomarkers at baseline but with more markers at year 1 of therapy. Both CMV and EBV DNA were associated with lower CD4/CD8 ratio, compatible with expansion of virus specific CD8⁺ T cells[6]. These diminished ratios are also important predictors of morbidity and mortality in PWH[38], yet the determinants of this relationship are unclear.

This study had a number of limitations. Because this was an observational study, we cannot establish a causal relationship between EBV and CMV DNA in PBMC and non-AIDS events. Also, some unmeasured variables might have confounded our analysis. For example, immune dysfunction from HIV infection alone could be driving both inflammation-related clinical events and CMV or EBV replication independently. The case-control design also makes it impossible to generate direct estimates of incident morbidity risk related to each assessed variable. In addition, the number of cases and controls with available samples

varied between time points and the small sample size within each event subgroup limited our power to observe associations between CMV DNA levels and specific non-AIDS events. Nevertheless, this study provides insights into the association between CMV and EBV DNA and non-AIDS clinical outcomes. Carefully designed clinical trials aimed at suppressing CMV and EBV replication among PWH are needed to confirm our findings and may help clarify the mechanisms between inflammation and related clinical events and might be important in improving the health of PWH who are co-infected with CMV and EBV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of Interest

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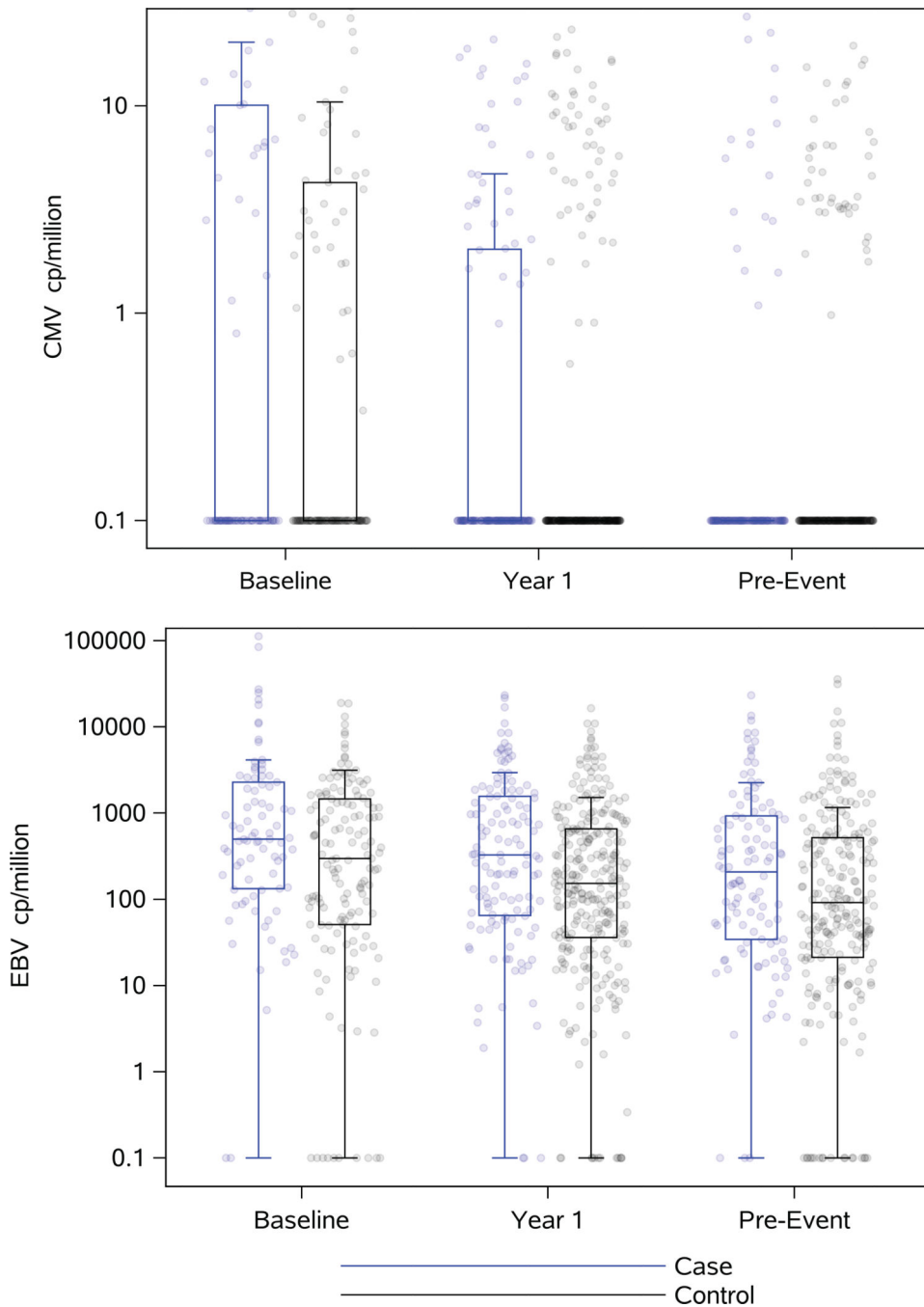


Figure 1: Levels of CMV and EBV DNA per million of mononuclear cells at each time point (baseline (case: N=85, control: N=147), year 1 (case: N=128, control: N=276) and pre-event (case: N=110, control: N=237)) for cases (in blue) and controls (black). Boxplots show medians and interquartile ranges. Lower limit of detection is 0 copies/million, represented as 0.1 copies/million due to log-transformation.

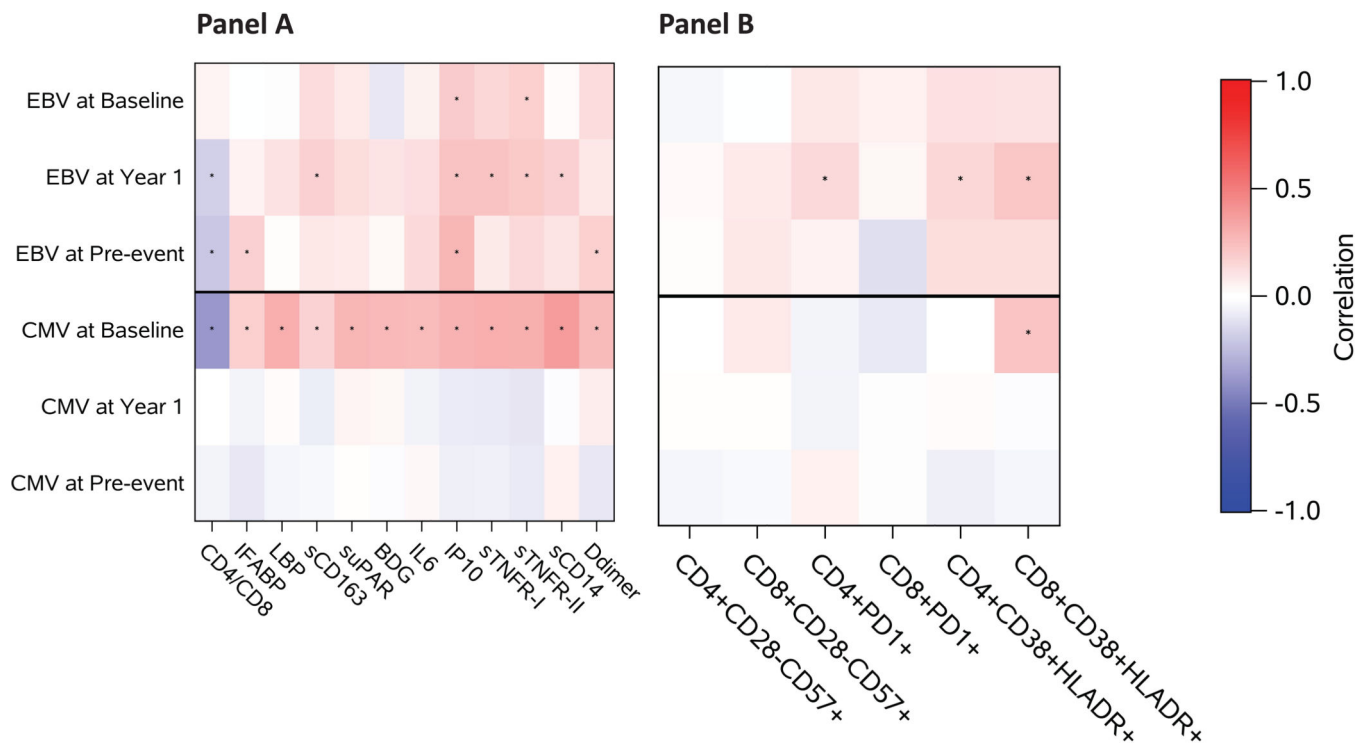


Figure 2.

Heatmap showing correlation coefficients between EBV and CMV DNA at each time point with various soluble (A) and cellular (B) markers of inflammation, immune activation, and gut damage among controls at baseline (A: N=125–147, B: N=110), year 1 (A: N=254–276, B: N=267), and pre-event (A: N=215–237, B: N=184). * indicated statistical significance at $p < 0.05$.

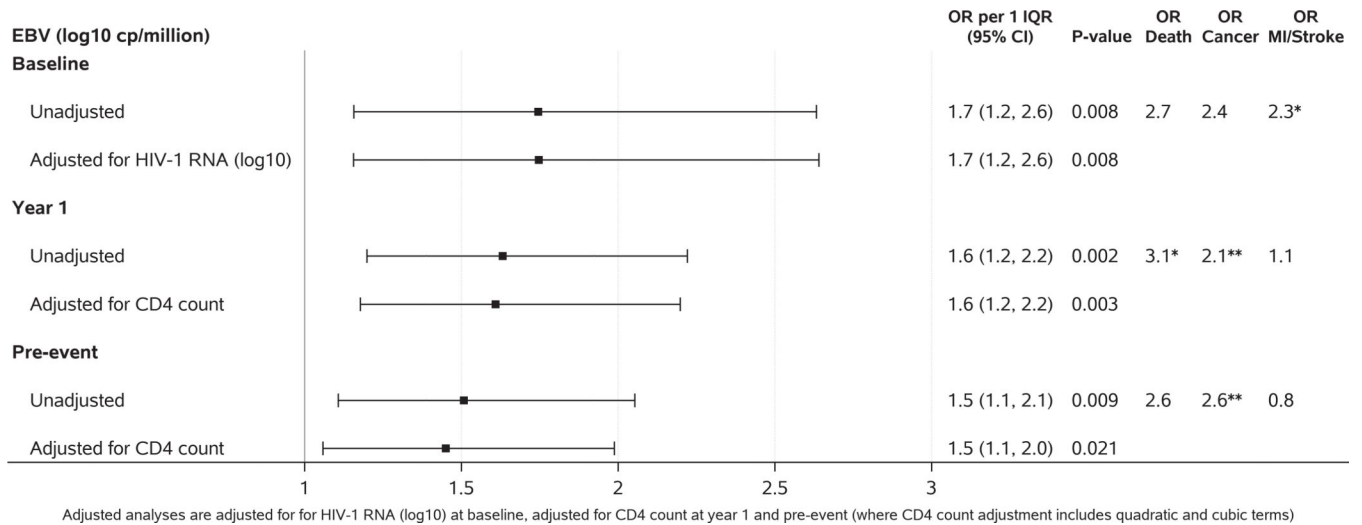
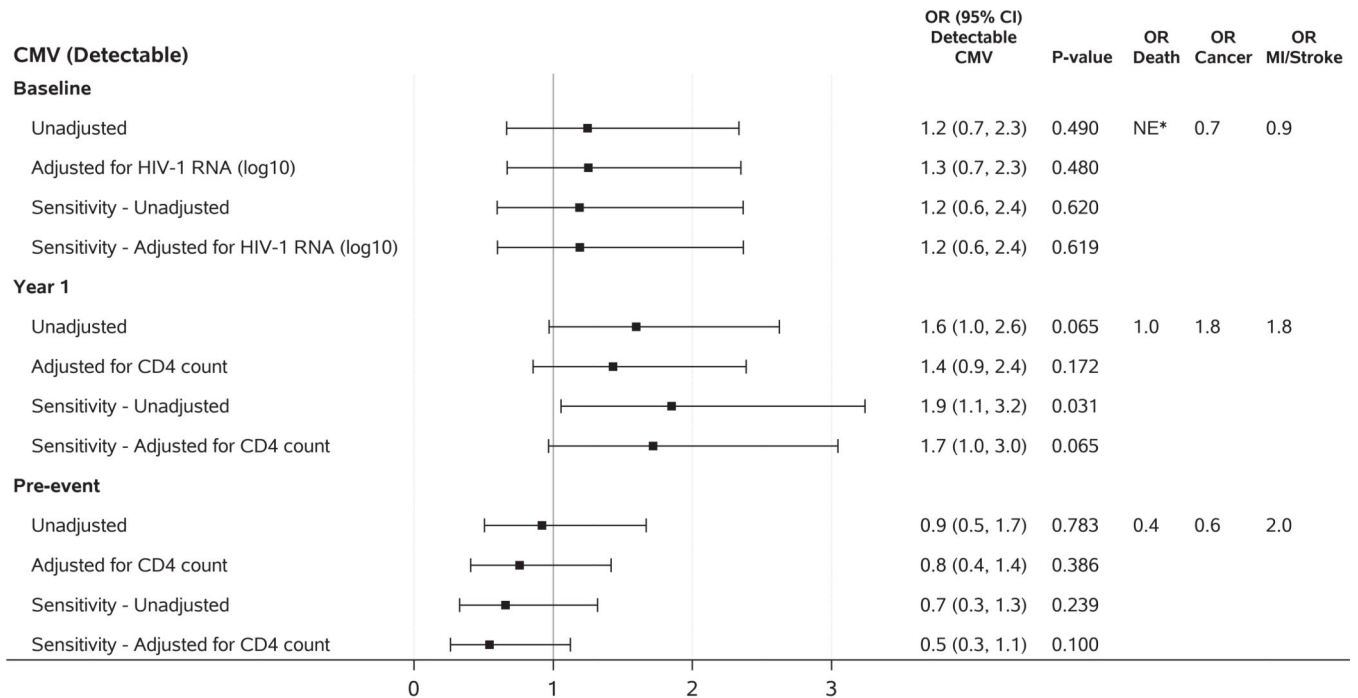


Figure 3. Associations between levels of EBV DNA and subsequent non-AIDS event at baseline (case: N=85, control: N=147), year 1 (case: N=128, control: N=276) and pre-event (case: N=110, control: N=237). Figure shows unadjusted analysis and analysis adjusted for HIV RNA (log₁₀) at baseline and for CD4 count at year 1 and pre-event (where CD4 count adjustment includes quadratic and cubic terms). * indicated statistical significance at p<0.05 while ** indicated p<0.01.



Adjusted analyses are adjusted for HIV-1 RNA (log₁₀) at baseline, adjusted for CD4 count at year 1 and pre-event (where CD4 count adjustment includes quadratic and cubic terms)

Sensitivity analyses excludes participants with undetectable CMV DNA results and low cell yields

*NE - Not estimable

Figure 4.

Associations between presence of detectable CMV DNA and subsequent non-AIDS event at baseline (case: N=85, control: N=147), year 1 (case: N=128, control: N=276) and pre-event (case: N=110, control: N=237). Analysis adjusted for HIV RNA (log₁₀) at baseline, adjusted for CD4 count at year 1 and pre-event (where CD4 count adjustment includes quadratic and cubic terms). Sensitivity analyses exclude participants with low cell yield. *NE – Not estimable. Using a post-hoc Cochran-Mantel-Haenszel test, there was an increased risk of death for those with detectable CMV DNA at baseline [odds ratio (95% CI) = 9.0 (1.9, 42.6), p=0.002, not shown in the figure].

Table 1:

Baseline Characteristics

Characteristic		Case (N=140)	Control (N=305)	Total (N=445)
Age (at parent study entry)	Years, Median (IQR)	46 (40–53)	44 (39–50)	45 (39–50)
Regimens (by parent study)	ACTG 384 / ACTG 388	39 (28%)	84 (28%)	123 (28%)
	A5014 / A5095 / A5142	69 (49%)	158 (52%)	227 (51%)
	A5202	32 (23%)	63 (21%)	95 (21%)
Sex	Male, N (%)	118 (84%)	258 (85%)	376 (84%)
	Female, N (%)	22 (16%)	47 (15%)	69 (16%)
Race/ethnicity	White Non-Hispanic, N (%)	72 (51%)	148 (49%)	220 (49%)
	Black Non-Hispanic, N (%)	52 (37%)	87 (29%)	139 (31%)
	Hispanic, N (%)	15 (11%)	59 (19%)	74 (17%)
	Others, N (%)	1 (1%)	11 (3%)	12 (3%)
Baseline CD4	Cells/ul, Median (IQR)	211 (89–341)	222 (76–335)	219 (77–336)
Baseline log ₁₀ HIV RNA	Copies/mL, Median (IQR)	4.8 (4.4–5.3)	4.8 (4.4–5.4)	4.8 (4.4–5.4)
Chronic Hepatitis B/C	Yes, N (%)	36 (26%)	34 (11%)	70 (16%)
Injection Drug Use	Yes, N (%)	20 (14%)	30 (10%)	50 (11%)
Waist-to-hip Ratio	Median (IQR)	0.92 (0.89–0.96)	0.92 (0.88–0.97)	0.92 (0.89–0.97)
History of Diabetes	Yes, N (%)	11 (8%)	15 (5%)	26 (6%)
History of Hypertension	Yes, N (%)	46 (33%)	55 (18%)	101 (23%)
Use of antihypertensive or lipid lowering agents	Yes, N (%)	32 (23%)	39 (13%)	71 (16%)
Current or Past Smoker	Yes, N (%)	105 (75%)	168 (55%)	273 (61%)
Family history of MI	Yes, N (%)	30 (21%)	45 (15%)	75 (17%)

IQR: interquartile range

See supplement for ACTG study details and regimens.