# Epstein–Barr virus and cytomegalovirus co-infections and mortality risk in patients with HIV-associated cryptococcal meningitis: a post-hoc analysis of a prospective nested cohort in the AMBITION-cm randomised controlled trial

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## Summary

**Background** HIV-associated cryptococcal meningitis case fatality remains greater than 25%. Co-prevalent infections might contribute to poor outcomes. We aimed to ascertain the prevalence and the clinical significance of Epstein–Barr virus (EBV) and cytomegalovirus co-infections in patients with cryptococcal meningitis to guide potential therapeutic interventions.

**Methods** We conducted a post-hoc analysis of a prospective cohort using plasma and cerebrospinal fluid (CSF) samples collected in the AMBITION-cm randomised trial. AMBITION-cm was done at seven hospital sites across five African countries (Botswana, Malawi, South Africa, Uganda, and Zimbabwe). The primary endpoint of the trial was all-cause mortality at 10 weeks. Quantitative PCR (qPCR) was used to measure baseline cytomegalovirus and EBV viral loads in these samples. Baseline demographic and clinical data including antiretroviral therapy status, and laboratory data including CD4 cell count, CSF white cell count, protein, glucose, and quantitative cryptococcal culture were captured in real time via an electronic medical records system. We assessed the prevalence of cytomegalovirus plasma viraemia and EBV plasma viraemia, and CNS co-infections, associations between cytomegalovirus and EBV co-infection status and baseline covariates, and associations with 2-week and 10-week mortality.

**Findings** Between Jan 31, 2018, and Feb 18, 2021, among 811 participants enrolled, 60% were male, median age was 37 years (IQR 32–43), and median baseline CD4 count was 27 cells per µL (IQR 10–58). Cytomegalovirus plasma viraemia was present in 395 (49%) of 804 participants and EBV plasma viraemia was present in 585 (73%) participants. 39 (5%) of 707 participants had detectable cytomegalovirus in the CSF and 191 (27%) of 708 participants had detectable EBV. Cytomegalovirus plasma viraemia was associated with lower CD4 cell counts, less CSF inflammation, and higher CSF fungal burdens. Conversely, EBV plasma viraemia was associated with higher CD4 cell counts and more CSF inflammation. At 2 and 10 weeks, the risk of mortality was two times higher in participants with high-level cytomegalovirus plasma viraemia (≥1000 copies per mL) than in participants without cytomegalovirus plasma viraemia (adjusted odds ratio 2·31 [95% CI 1·12–4·75] at 2 weeks; 2·44 [1·33–4·45] at 10 weeks). EBV coinfections were not associated with increased mortality.

**Interpretation** These data indicate that cytomegalovirus might be an important copathogen in this context, and that cytomegalovirus viraemia represents a potentially modifiable risk factor to reduce mortality among adults with HIV-associated cryptococcal meningitis. Interventional trials are now required and planned to determine whether treatment of cytomegalovirus viraemia improves outcomes in advanced HIV disease.

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## Introduction

*Cryptococcus* remains the most common cause of HIV-associated meningitis globally, accounting for approximately 19% of all AIDS-related deaths.[1] In Africa, case fatality is 24–45%, even in the context of clinical trials;[2–5] undiagnosed co-prevalent opportunistic infections might contribute to poor outcomes.[6–8] The epidemiology of opportunistic viral infections in Africa, particularly CNS infections, has been poorly characterised due to the shortage of sensitive diagnostics.[9] The few studies that have performed PCR testing on cerebrospinal fluid (CSF) from individuals with HIV presenting with symptoms of CNS infections have found high rates of Epstein–Barr virus (EBV) detection, relatively frequent cytomegalovirus detection, and evidence for co-infection with two or more potential pathogens in up to 39% of cases.[10–12] The data

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## Research in context

### Evidence before this study

Mortality due to HIV-associated cryptococcal meningitis remains high (24–45%), even in the context of clinical trials; undiagnosed opportunistic infections might contribute to poor outcomes. Cytomegalovirus viraemia is common in the context of HIV-associated cryptococcal meningitis and has been shown to be associated with increased risk of death. The prognostic significance of cytomegalovirus CNS infections and Epstein–Barr virus (EBV) co-infections are poorly understood. We searched PubMed from database inception to Dec 9, 2024, for articles investigating cytomegalovirus or EBV co-infections among adults with HIV-associated cryptococcal meningitis using the terms: "(CMV OR Cytomegalovirus OR EBV OR Epstein–Barr virus)" AND "cryptococcal meningitis", without any language or date restrictions. This search yielded 148 studies, of which two studies described the prevalence of cytomegalovirus plasma viraemia in the context of HIV-associated cryptococcal meningitis, and three studies which described the prevalence of cytomegalovirus and EBV CNS infections among adults with suspected HIV-associated neurological infections. All studies were conducted at a single site and included relatively small sample sizes.

### Added value of this study

This is the largest multi-country study to describe the prevalence of cytomegalovirus and EBV co-infections in the context of HIV-associated cryptococcal meningitis. Half of all participants had evidence of cytomegalovirus viraemia, and EBV co-infections occurred in nearly three-quarters of the cohort. Cytomegalovirus and EBV co-infections were associated with contrasting clinical phenotypes. Cytomegalovirus viraemia was strongly and independently associated with greater than double the odds of mortality at 10 weeks. A greater proportion of participants with cytomegalovirus CNS co-infection died than did those without, but this result was not statistically significant. EBV co-infections were not associated with worse outcomes.

### Implications of all the available evidence

Cytomegalovirus viraemia represents a modifiable risk factor to potentially improve survival among adults with cryptococcal meningitis. Interventional trials are now required to determine whether treatment of cytomegalovirus impacts outcomes in advanced HIV disease.

available suggest that EBV co-infection is common in cryptococcal meningitis, with EBV detected in the CSF of 12 (25%) of 48 patients with cryptococcal meningitis undergoing detailed molecular diagnostic testing in Zambia[10] and 40 (43%) of 93 patients in Uganda.[11,12] Cytomegalovirus co-detection in the CSF of patients with cryptococcal meningitis was less common in these studies, occurring in 1–5% of participants.[10–12] Conversely, plasma cytomegalovirus viraemia is common among adults with HIV-associated cryptococcal meningitis (36–52%).[7,8] The prevalence of EBV viraemia in adults with HIV-associated cryptococcal meningitis has not been previously described.

Cytomegalovirus viraemia has been reported to be associated with increased mortality in the context of HIV-associated cryptococcal meningitis.[7,8] The clinical implications and prognostic significance of cytomegalovirus CNS co-infection, EBV viraemia, or EBV CNS co-infection are less well understood. Both EBV and cytomegalovirus might cause neurological damage by direct invasion of the brain cells[13] or trigger immune-mediated damage,[14] might amplify CNS damage,[15] and impair clearance of other pathogens.[16] Alternatively, detection of EBV or cytomegalovirus by PCR might be a non-pathogenic marker of profound HIV-mediated immunosuppression, or a bystander effect of CNS inflammation with reactivated latent virus shed from activated leukocytes and other cell types during meningitis infection.[9,12]

To ascertain the clinical significance of EBV and cytomegalovirus co-infections in patients with cryptococcal meningitis and guide potential therapeutic interventions, we aimed to determine the prevalence of EBV and cytomegalovirus viraemia in plasma, and CNS co-infections, in a cohort of adults with HIV-associated cryptococcal meningitis from five African countries and explored associations between EBV and cytomegalovirus co-infections and mortality.

## Methods

### Study design and participants

In this post-hoc analysis, we analysed baseline plasma and CSF samples and clinical data collected prospectively from adults (aged ≥18 years) with confirmed HIV-associated cryptococcal meningitis (CSF cryptococcal antigen positive) recruited as part of AMBITION-cm, a multi-site, phase 3 randomised, controlled trial, which investigated the use of an initial treatment regimen based on a single high dose of liposomal amphotericin B (10 mg per kg of bodyweight) plus 14 days of fluconazole and flucytosine compared with the previously recommended WHO-recommended standard of care (amphotericin B deoxycholate 1 mg/kg per day plus flucytosine for 7 days, followed by high-dose fluconazole for 7 days).[5] The primary endpoint was all-cause mortality at 10 weeks. 844 participants were randomly assigned in Botswana, Malawi, South Africa, Uganda, and Zimbabwe.

The AMBITION-cm protocol and associated substudies were approved by the London School of Hygiene and Tropical Medicine Research Ethics Committee (14355) and by the relevant ethics committees and national regulatory agencies overseeing the trial sites. Written

informed consent was obtained for all participants which included consent for use of samples for future studies.

### Procedures

Baseline demographic and clinical data including antiretroviral therapy (ART) status, and laboratory data including CD4 cell count, CSF white cell count, protein, glucose, and quantitative cryptococcal culture were recorded in real time via an electronic medical records system. Participants were followed up for 10 weeks.

All AMBITION-cm participants with baseline plasma or CSF available for testing were included in this substudy. Stored baseline plasma and CSF samples from the AMBITION-cm trial were retrospectively tested at City St George's, University of London (London, UK). DNA extraction from plasma and CSF was done using the Qiagen QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Multiplex quantitative PCR (qPCR) reactions were assembled with TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), which contains AmpliTaq Gold DNA Polymerase, reaction buffer, dNTPs mix (with dUTP), and $MgCl_2$; and 0·3 µM primers, 0·15 µM probes, and uracil-DNA glycosylase, 7 µL of DNA template, in a final volume of 20 µL. For cytomegalovirus detection, primers DNApol_F (5′-GCATGCGCGAGTGTCAAGAC-3′) and DNApol_R (5′-GTTACTTTGAGCGCCATCTGTTCCT-3′), and probe DNApol_probe (FAM-TGCGCCGTATGCTGCTCGACA–BHQ1) were used. For EBV detection, primers EBV_F (5′-CCG GTGTGTTCGTATATGGAG-3′) and EBV_R (5′-GGGAGACGACTCAATGGTGTA-3′), and probe EBV_probe (HEX-TGCCCTTGCTATTCCACAATGT CGT–BHQ1) were used. The qPCR was performed using a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve for cytomegalovirus_DNApol was generated using five 10-fold dilutions from $10^4$ copies per µL of control plasmid (DNApol fragment cloned in pUC57; GenScript, Piscataway, NJ, USA). The standard curve for EBV was generated using six 10-fold dilutions starting with $3\times10^4$ IU/µL of EBV DNA extracted from the WHO International standard for EBV nucleic acid amplification techniques.

Cytomegalovirus viral load measurements are reported in copies per mL, the assay limit of quantitation was 15·42 copies per mL. EBV viral load measurements are reported in IU/mL, the assay limit of quantitation was 50·51 IU/mL. Laboratory staff processed coded samples and were masked to participant data.

### Statistical analysis

Baseline variables were summarised and the proportion of participants with cytomegalovirus and EBV plasma viraemia and CNS co-infection determined. Cytomegalovirus or EBV plasma viraemia was defined as detectable cytomegalovirus or EBV DNA in plasma. CNS co-infection was defined as detectable cytomegalovirus or EBV DNA in CSF. Baseline variables were compared according to cytomegalovirus and EBV co-infection status using the $\chi^2$ test for proportions, and Mann–Whitney *U* test or the Student's *t*-test for continuous data.

The primary outcome was all-cause mortality at 10 weeks with all-cause mortality at 2 weeks as a secondary outcome. Univariable and multivariable logistic regression models were used to evaluate associations between cytomegalovirus and EBV co-infection status, and mortality at 2 weeks and 10 weeks. In the adjusted regression models, age, sex, study site, and baseline CD4 cell count were included a priori. Covariates considered to be potentially on the causal pathway between cytomegalovirus and EBV co-infections and mortality were not included in the regression models (appendix p 1). A likelihood ratio test was conducted to test for an interaction between ART status and cytomegalovirus viraemia co-infection status using an adjusted logistic regression model.

See Online for appendix

To investigate the dose–response relationship between cytomegalovirus viral load and outcome, we stratified participants with cytomegalovirus co-infections into low-level infection (<1000 copies per mL) and high-level infection (≥1000 copies per mL) groups, a pre-emptive treatment threshold frequently used in haemopoietic stem cell transplant cohorts.[17] We utilised logistic regression models to investigate a dose–response relationship, and in addition we used a generalised linear model with an identity link function to calculate the risk difference in mortality at 2 weeks and 10 weeks between participants with high-level cytomegalovirus viraemia versus no cytomegalovirus viraemia. We also analysed plasma cytomegalovirus viral load as a continuous variable. Since no recognised threshold exists for high-level EBV infection, the primary EBV analyses were based on a dichotomous variable of presence or absence of EBV viraemia or EBV CNS co-infection only. In an exploratory secondary analysis, we also compared outcomes between participants with low-level EBV co-infections (<1000 EBV copies per IU/mL) and high-level co-infections (≥1000 EBV copies per IU/mL). Kaplan–Meier curves were plotted to 10 weeks according to EBV and cytomegalovirus status.

In the presence of missing data, only complete cases were used for multivariable analysis. All analyses were performed in Stata (version 16). p values were not adjusted for multiple comparisons.

### Role of the funding source

The funders had no role in the study design, analysis, interpretation, or decision to publish the manuscript.

## Results

We performed cytomegalovirus and EBV qPCR viral load testing for 811 Black African adults with HIV-associated

cryptococcal meningitis; 804 participants had baseline plasma samples available for analysis, and 708 participants had baseline CSF samples. 701 participants had paired plasma and CSF samples. The median age was 37 years (IQR 32–43), and 490 (60%) of 811 participants were male. The median CD4 count was 27 cells per µL (IQR 10–58), and 521 (64%) of 811 participants were ART experienced at presentation.

395 (49%) of 804 participants had cytomegalovirus plasma viraemia, with a median plasma viral load of 103 copies per mL (IQR 39–404). 59 (7%) of 804 participants had cytomegalovirus plasma viraemia of 1000 copies per mL or higher. 585 (73%) of 804 participants had EBV plasma viraemia, with a median plasma viral load of 877 IU/mL (IQR 157–4637). 283 (35%) of 804 participants had EBV viraemia (≥1000 IU/mL).

CNS co-infections were less common; 39 (5%) of 707 participants had detectable cytomegalovirus in CSF, and 191 (27%) of 708 participants had detectable EBV in the CSF. The median cytomegalovirus CSF viral load was 127 copies per mL (IQR 29–1583), and 12 (2%) of 707 participants had a CSF cytomegalovirus viral load of

| | No cytomegalovirus viraemia (n=409) | Cytomegalovirus viraemia (n=395) | p value | No cytomegalovirus CNS co-infection (n=668) | Cytomegalovirus CNS co-infection (n=39) | p value |
|---|---|---|---|---|---|---|
| Male | 247 (60·4%) | 239 (60·5%) | 0·97 | 398 (59·6%) | 24 (61·5%) | 0·81 |
| Female | 162 (39·6%) | 156 (39·5%) | .. | 270 (40·4%) | 15 (38·5%) | .. |
| Age, years | 37 (32–43) | 37 (31–43) | 0·74 | 37 (32–43) | 36 (31–42) | 0·73 |
| ART experienced | 266 (65·0%) | 250 (63·3%) | 0·61 | 454 (65·8%) | 23 (57·5%) | 0·48 |
| Baseline CD4 count (cells per µL) | 33 (12–68) | 23 (9–51) | 0·0001 | 28 (11–63) | 27 (9–42) | 0·35 |
| Fever at baseline | 132 (32·4%) | 138 (35·2%) | 0·39 | 232 (34·9%) | 13 (33·3%) | 0·84 |
| Glasgow Coma Scale <15 | 112 (27·4%) | 117 (29·6%) | 0·48 | 198 (29·6%) | 15 (38·5%) | 0·24 |
| Seizures | 35 (8·6%) | 51 (13·0%) | 0·043 | 72 (10·8%) | 6 (15·4%) | 0·38 |
| CSF white cell count (cells per µL) | 10 (4–77) | 4 (2–45) | 0·0005 | 5 (3–55) | 6 (1–75) | 0·48 |
| CSF protein (g/dL) | 0·9 (0·5–1·6) | 0·8 (0·4–1·3) | 0·013 | 0·9 (0·4–1·4) | 0·8 (0·5–1·7) | 0·60 |
| CSF fungal burden (CFU per mL) | 30 000 (250–350 000) | 70 000 (1100–465 000) | 0·0011 | 45 750 (385–397 500) | 85 000 (5700–500 000) | 0·18 |

Data are n (%) or median (IQR). Some percentages do not sum to 100 due to rounding. For categorical variables, the p value was calculated using the $\chi^2$ test if there were five participants or more in each category, or using Fisher's exact test if there were fewer than five participants. For non-categorical variables p values were calculated using the Mann–Whitney *U* test throughout due to the non-parametric distribution of all data. Three participants with cytomegalovirus plasma viraemia and one participant without cytomegalovirus plasma viraemia were missing baseline data on fever and seizures. Eight participants with cytomegalovirus plasma viraemia, 11 participants without cytomegalovirus plasma viraemia, two participants with cytomegalovirus CNS co-infection, and 13 participants without cytomegalovirus CNS co-infection were missing a CSF white cell count. 17 participants with cytomegalovirus plasma viraemia, 13 participants without cytomegalovirus plasma viraemia, two participants with cytomegalovirus CNS co-infection, and 15 participants without cytomegalovirus CNS co-infection were missing CSF protein counts. There was no association with baseline cytomegalovirus viraemia status and receipt of tuberculosis treatment during the trial: 55 (13%) of 409 participants without cytomegalovirus viraemia were treated for tuberculosis versus 55 (14%) of 395 participants with cytomegalovirus viraemia (p=0·84). ART=antiretroviral therapy. CSF=cerebrospinal fluid. CFU=colony-forming units.

***Table 1:*** **Baseline characteristics of 811 adults with HIV-associated cryptococcal meningitis stratified by cytomegalovirus viraemia co-infection status and cytomegalovirus CNS co-infection status**

| | No EBV viraemia (n=219) | EBV viraemia (n=585) | p value | No EBV CNS co-infection (n=517) | EBV CNS co-infection (n=191) | p value |
|---|---|---|---|---|---|---|
| Male | 126 (58%) | 360 (62%) | 0·30 | 310 (60%) | 112 (59%) | 0·75 |
| Female | 93 (43%) | 225 (39%) | .. | 207 (40%) | 79 (41%) | .. |
| Age, years | 35 (30–41) | 38 (32–44) | 0·0003 | 36 (32–43) | 38 (33–43) | 0·15 |
| ART experienced | 141 (64%) | 375 (64%) | 0·94 | 327 (63%) | 135 (71%) | 0·065 |
| Baseline CD4 count (cells per µL) | 20 (8–49) | 30 (11–64) | 0·0017 | 23 (9–52) | 42 (21–84) | <0·0001 |
| Fever at baseline | 80 (37%) | 190 (33%) | 0·28 | 166 (32%) | 79 (42%) | 0·021 |
| Glasgow Coma Scale <15 | 48 (22%) | 181 (31%) | 0·012 | 147 (28·4%) | 66 (35·0%) | 0·11 |
| Seizures | 19 (8·7%) | 67 (11·5%) | 0·26 | 54 (11%) | 24 (13%) | 0·42 |
| CSF white cell count (cells per µL) | 4 (2–39) | 6 (4–70) | 0·013 | 4 (2–38) | 21 (4–110) | <0·0001 |
| CSF protein (g/dL) | 0·7 (0·4–1·3) | 0·9 (0·5–1·5) | 0·0043 | 0·8 (0·4–1·3) | 1·1 (0·6–2·0) | <0·0001 |
| CSF fungal burden (CFU per mL) | 46 500 (365–495 000) | 45 500 (415–370 000) | 0·48 | 64 000 (410–465 000) | 24 000 (400–315 000) | 0·055 |

Data are n (%) or median (IQR). Some percentages do not sum to 100 due to rounding. For categorical variables, the p value was calculated using the $\chi^2$ test if there were five participants or more in each category, or using Fisher's exact test if there were fewer than five participants. For non-categorical variables p values were calculated using the Mann–Whitney *U* test throughout due to the non-parametric distribution of all data. Three participants with EBV plasma viraemia, one participant without EBV plasma viraemia, one participant with EBV CNS co-infection, and two participants without EBV CNS co-infection were missing baseline data on fever and seizures. 14 participants with EBV plasma viraemia, five participants without EBV plasma viraemia, two participants with EBV CNS co-infection, and 13 participants without EBV CNS co-infection were missing a CSF white cell count. 22 participants with EBV plasma viraemia, eight participants without EBV plasma viraemia, three participants with EBV CNS co-infection, and 14 participants without EBV CNS co-infection were missing CSF protein counts. ART=anti-retroviral therapy. CSF=cerebrospinal fluid. CFU=colony-forming units. EBV=Epstein–Barr virus.

***Table 2:*** **Baseline characteristics of 811 adults with HIV-associated cryptococcal meningitis stratified by EBV viraemia co-infection status and EBV CNS co-infection status**

1000 copies per mL or higher. The median CSF EBV viral load was 303 IU/mL (IQR 145–1735), and 62 (9%) of 708 participants had a CSF EBV viral load of 1000 IU/mL or higher.

Of 700 participants with paired plasma and CSF cytomegalovirus viral load results, 27 (8%) of 338 participants with cytomegalovirus plasma viraemia had evidence of cytomegalovirus CNS co-infection. The median plasma cytomegalovirus viral load was higher (p=0·0006) among participants with evidence of cytomegalovirus CNS co-infection (347 copies per mL) than among those without cytomegalovirus CNS co-infection (94 copies per mL). Among 54 participants with high-level cytomegalovirus viraemia (≥1000 copies per mL), seven (13%) had concurrent cytomegalovirus CNS co-infection. 12 participants had cytomegalovirus CNS co-infection in the absence of plasma cytomegalovirus viraemia.

Of 701 participants with paired plasma and CSF EBV viral load results, a third of participants with EBV viraemia had EBV CNS co-infection (166 [32%] of 511 participants). The median plasma EBV viral load was 3052 IU/mL in participants with EBV CNS co-infection versus 429 IU/mL in those with EBV viraemia without EBV CNS co-infection (p<0·0001). EBV CNS co-infection in the absence of plasma EBV viraemia was observed in 25 participants. More than a third of the cohort (298 [37%] of 804 participants) had evidence of dual EBV and cytomegalovirus viraemia, and ten (1%) of 707 participants had both EBV and cytomegalovirus detectable in their CSF.

Participants with cytomegalovirus plasma viraemia had a lower median baseline CD4 count (23 *vs* 33 cells per µL; p=0·0001), less CSF inflammation (median CSF white cell count 4 *vs* 10 cells per µL; p=0·0005), lower median CSF protein (0·8 *vs* 0·9 g/dL; p=0·01), and significantly higher CSF fungal burdens (median cryptococcal quantitative fungal culture 70000 *vs* 30000 colony-forming units [CFU] per mL; p=0·001) than participants without cytomegalovirus plasma viraemia. Similar findings were observed when comparing participants with cytomegalovirus CNS co-infection with participants without cytomegalovirus CNS co-infection but the differences between groups were not statistically significant (table 1).

Conversely, participants with EBV plasma viraemia had a higher median age (38 years *vs* 35 years; p=0·0003), a higher median baseline CD4 count (30 *vs* 20 cells per µL; p=0·002), and more inflammatory CSF profiles (median CSF white cell count 6 *vs* 4 cells per µL; p=0·01), and median CSF protein (0·9 *vs* 0·7 g/dL; p=0·004) compared with participants without EBV plasmaviraemia. Similarly, participants with EBV CNS co-infection had more CSF inflammation than participants without EBV CNS co-infection (median CSF white cell count 21 *vs* 4 cells per µL; p<0·001). CSF fungal burden was lower among participants with EBV CNS co-infection than those without EBV CNS co-infection (median cryptococcal quantitative fungal culture 24000 *vs* 64000 CFU per mL; p=0·05; table 2).

At 2 weeks, 104 (13%) of 811 participants had died, and at 10 weeks, 218 (27%) participants had died. Mortality was higher among participants with cytomegalovirus co-infections than participants without. At 2 weeks, 59 (15%) of 395 participants with cytomegalovirus viraemia had died versus 44 (11%) of 409 participants without cytomegalovirus viraemia (p=0·08); at 10 weeks, 119 (30%) of 395 participants with cytomegalovirus viraemia had died versus 98 (24%) of 409 participants without cytomegalovirus viraemia (p=0·05; figure 1).

Mortality at 10 weeks was significantly higher among participants with high-level cytomegalovirus viraemia (≥1000 copies per mL; 25 [42%] of 59 participants) than in participants with low-level cytomegalovirus viraemia (<1000 copies per mL; 94 [28%] of 326 participants) and participants with no cytomegalovirus viraemia (98 [24%] of 409 participants; p=0·01). Mortality at 2 weeks was also significantly higher among participants with high-level cytomegalovirus viraemia than those without (13 [22%] of 59 participants with high-level cytomegalovirus viraemia *vs* 44 [11%] of 409 participants without cytomegalovirus viraemia; p=0·016; table 3), suggesting a dose–response relationship between cytomegalovirus viraemia and mortality. Compared with participants without cytomegalovirus viraemia, the unadjusted risk difference for mortality at 2 weeks among participants with high-level cytomegalovirus viraemia (≥1000 copies per mL) was 11·3% (95% CI 0·3–22·3; p=0·044), and for mortality at 10 weeks, the unadjusted risk difference was 18·0% (5·1–31·7; p=0·007).

Using multivariate logistic regression modelling, high-level cytomegalovirus plasma viraemia (≥1000 copies per mL) was strongly and independently associated with greater than double the odds of mortality at 2 weeks (adjusted odds ratio [aOR] 2·31 [95% CI 1·12–4·75];

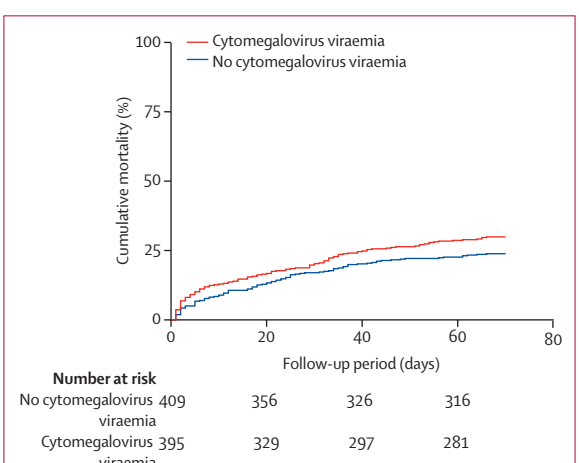


***Figure 1:*** **Cumulative mortality at 10 weeks in adults with cryptococcal meningitis, stratified by presence or absence of cytomegalovirus plasma viraemia (n=811)**

| | Mortality, n/N (%) | cOR (95% CI) | p value | aOR* (95% CI) | p value |
|---|---|---|---|---|---|
| **Week 2** | | | | | |
| Total cohort | 104/811 (13%) | .. | .. | .. | .. |
| No cytomegalovirus viraemia | 44/409 (11%) | 1 (ref) | .. | 1 (ref) | .. |
| Cytomegalovirus viraemia | 59/395 (15%) | 1·32 (0·97–1·79) | 0·077 | 1·42 (0·92–2·21) | 0·11 |
| Low-level cytomegalovirus viraemia (<1000 copies per mL) | 46/336 (14%) | 1·32 (0·85–2·04) | 0·22 | 1·29 (0·81–2·04) | 0·28 |
| High-level cytomegalovirus viraemia (≥1000 copies per mL) | 13/59 (22%) | 2·34 (1·17–4·68) | 0·016 | 2·31 (1·12–4·75) | 0·023 |
| **Week 10** | | | | | |
| Total cohort | 218/811 (27%) | .. | .. | .. | .. |
| No cytomegalovirus viraemia | 98/409 (24%) | 1 (ref) | .. | 1 (ref) | .. |
| Cytomegalovirus viraemia | 119/395 (30%) | 1·37 (1·00–1·87) | 0·049 | 1·30 (0·94–1·83) | 0·11 |
| Low-level cytomegalovirus viraemia (<1000 copies per mL) | 94/336 (28%) | 1·23 (0·88–1·71) | 0·21 | 1·17 (0·82–1·66) | 0·38 |
| High-level cytomegalovirus viraemia (≥1000 copies per mL) | 25/59 (42%) | 2·33 (1·34–4·10) | 0·003 | 2·44 (1·33–4·45) | 0·004 |

Inclusion of baseline ART status or AMBITION-cm treatment group did not alter the model findings. We assessed for multicollinearity among predictors using variance inflation factors, with a threshold of 10 indicating potential concern. The variance inflation factors for all predictors were below 1·7, indicating no significant multicollinearity. Goodness-of-fit was assessed using the Hosmer–Lemeshow test. The Hosmer–Lemeshow test indicated acceptable model fit (p>0·05 for each). The unadjusted plasma cytomegalovirus models included 804 complete cases records. The adjusted plasma cytomegalovirus models included 767 complete cases records. The unadjusted CSF cytomegalovirus models included 707 complete cases records. The adjusted CSF cytomegalovirus models included 677 complete cases records. cOR=crude unadjusted odds ratio. aOR=adjusted odds ratio. ART=antiretroviral therapy. CSF=cerebrospinal fluid. *Adjusted for age (continuous variable), baseline CD4 cell count (continuous variable), sex, and study site.

***Table 3:*** **Multivariate analysis of the association between cytomegalovirus viraemia and mortality at 2 and 10 weeks using logistic regression modelling**

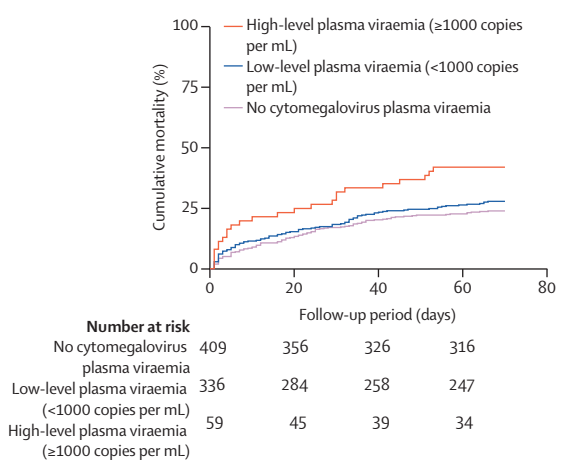


***Figure 2:*** **Cumulative mortality at 10 weeks in adults with cryptococcal meningitis, stratified by cytomegalovirus viral load burden (n=811)**

p=0·02) and 10 weeks (aOR 2·44 [1·33–4·45]; p=0·004) after adjustment for age, sex, CD4 cell count, and study site when compared with those without cytomegalovirus viraemia (table 3, figure 2). There was no evidence that ART status acted as a confounder of the observed association between high-level cytomegalovirus viraemia and death, nor was there evidence of effect mediation between ART status and low-level or high-level cytomegalovirus viraemia status (p=0·41). There was no evidence that AMBITION-cm treatment group was a confounder of the observed association between high-level cytomegalovirus viraemia and death.

When cytomegalovirus viral load was analysed as a continuous variable, we found that for every 1000 copies per mL increase in plasma cytomegalovirus burden, odds of mortality at 2 weeks increased by 13% (OR 1·13 [95% CI 0·95–1·36]; p=0·17), and odds of mortality at 10 weeks increased by 27% (1·27 [1·08–1·49]; p=0·004). The observed independent association with increased mortality at 10 weeks remained after adjustment for age, sex, CD4 cell count, and study site (aOR 1·28 [1·07–1·52]; p=0·005).

Mortality at 10 weeks was similar between participants with cytomegalovirus CNS co-infection (12 [31%] of 39 participants) and without cytomegalovirus CNS co-infection (184 [28%] of 668 participants; p=0·66). At 2 weeks, eight (21%) of 39 participants with cytomegalovirus CNS co-infection had died versus 86 (13%) of 668 participants without cytomegalovirus CNS co-infection (p=0·18). Mortality at 10 weeks was higher among participants with high-level cytomegalovirus CNS co-infection (≥1000 copies per mL; four [33%] of 12 participants) than among participants with low-level cytomegalovirus CNS co-infection (<1000 copies per mL; eight [30%] of 27 participants) and participants without cytomegalovirus CNS co-infection (184 [28%] of 668 participants), but the difference was not statistically significant (p=0·38; table 4). Following adjustment for age, sex, CD4 cell count, and study site, in participants with high-level cytomegalovirus CNS co-infection (≥1000 copies per mL) versus participants without cytomegalovirus CNS co-infection, the adjusted odds ratio for mortality at 2 weeks was 1·84 (95% CI 0·38–8·91) and 1·76 (0·50–6·22) at 10 weeks (table 4). The mortality rate at 10 weeks was similar among participants with EBV viraemia and those without EBV viraemia (162 [28%] of 585 participants *vs* 55 [25%] of 219 participants; p=0·46). The mortality rate at 2 weeks was also similar in participants with and without

| | Mortality, n/N (%) | cOR (95% CI) | p value | aOR * (95% CI) | p value |
|---|---|---|---|---|---|
| **Week 2** | | | | | |
| Total cohort | 104/811 (13%) | .. | .. | .. | .. |
| No cytomegalovirus CNS infection | 86/668 (13%) | 1 (ref) | .. | 1 (ref) | .. |
| Cytomegalovirus CNS infection | 8/39 (21%) | 1·75 (0·78–3·92) | 0·18 | 2·03 (0·87–4·70) | 0·10 |
| Low-level cytomegalovirus CNS infection (<1000 copies per mL) | 6/27 (22%) | 1·93 (0·76–4·93) | 0·17 | 2·10 (0·80–5·52) | 0·13 |
| High-level cytomegalovirus CNS infection (≥1000 copies per mL) | 2/12 (17%) | 1·35 (0·29–6·28) | 0·70 | 1·84 (0·38–8·91) | 0·45 |
| **Week 10** | | | | | |
| Total cohort | 218/811 (27%) | .. | .. | .. | .. |
| No cytomegalovirus CNS infection | 184/668 (28%) | 1 (ref) | .. | 1 (ref) | .. |
| Cytomegalovirus CNS infection | 12/39 (31%) | 1·17 (0·58–2·36) | 0·66 | 1·14 (0·54–2·42) | 0·72 |
| Low-level cytomegalovirus CNS infection (<1000 copies per mL) | 8/27 (30%) | 1·11 (0·48–2·57) | 0·81 | 0·95 (0·38–2·34) | 0·91 |
| High-level cytomegalovirus CNS infection (≥1000 copies per mL) | 4/12 (33%) | 1·31 (0·39–4·42) | 0·66 | 1·76 (0·50–6·22) | 0·38 |

Inclusion of baseline ART status or AMBITION-cm treatment group did not alter the model findings. We assessed for multicollinearity among predictors using variance inflation factors, with a threshold of 10 indicating potential concern. The variance inflation factors for all predictors were below 1·7, indicating no significant multicollinearity. Goodness-of-fit was assessed using the Hosmer–Lemeshow test. The Hosmer–Lemeshow test indicated acceptable model fit (p>0·05 for each). The unadjusted CNS cytomegalovirus models included 707 complete cases records. The adjusted CNS cytomegalovirus models included 677 complete cases records. cOR=crude unadjusted odds ratio. aOR=adjusted odds ratio. ART=antiretroviral therapy. CSF=cerebrospinal fluid. *Adjusted for age (continuous variable), baseline CD4 cell count (continuous variable), sex, and study site.

***Table 4:*** **Multivariate analysis of the association between cytomegalovirus CNS infection and mortality at 2 and 10 weeks using logistic regression modelling**

EBV (table 5). Following adjustment for age, sex, CD4 cell count, and study site, the odds ratio for mortality at 2 weeks for participants with EBV viraemia was 0·89 (95% CI 0·54–1·45; p=0·63) versus participants without EBV viraemia. Using the same model, results were similar at 2 weeks for participants with EBV CNS co-infection versus participants without EBV CNS co-infection (aOR 0·88 [0·51–1·51]; p=0·64; table 5). In our exploratory EBV dose–response analyses, no significant associations were identified between high-level EBV viraemia (EBV plasma viral load ≥1000 IU/mL) and mortality at 2 or 10 weeks compared with participants without EBV viraemia; nor between high-level EBV CNS co-infection (EBV CSF viral load ≥1000 IU/mL) and mortality at 2 or 10 weeks when compared with participants without EBV CNS co-infection (appendix p 2).

| | Mortality, n/N (%) | cOR (95% CI) | p value | aOR* (95% CI) | p value |
|---|---|---|---|---|---|
| **Week 2** | | | | | |
| Total cohort | 104/811 (13%) | .. | .. | .. | .. |
| No EBV viraemia | 30/219 (14%) | 1 (ref) | .. | 1 (ref) | .. |
| EBV viraemia | 73/585 (13%) | 0·90 (0·57–1·42) | 0·65 | 0·89 (0·54–1·45) | 0·63 |
| No EBV CNS infection | 72/517 (14%) | 1 (ref) | .. | 1 (ref) | .. |
| EBV CNS infection | 22/191 (12%) | 0·80 (0·48–1·34) | 0·40 | 0·88 (0·51–1·51) | 0·64 |
| **Week 10** | | | | | |
| Total cohort | 218/811 (27%) | .. | .. | .. | .. |
| No EBV viraemia | 55/219 (25%) | 1 (ref) | .. | 1 (ref) | .. |
| EBV viraemia | 162/585 (28%) | 1·14 (0·80–1·63) | 0·46 | 1·04 (0·71–1·53) | 0·83 |
| No EBV CNS infection | 145/517 (28%) | 1 (ref) | .. | 1 (ref) | .. |
| EBV CNS infection | 52/191 (27%) | 0·96 (0·66–1·39) | 0·83 | 0·98 (0·66–1·47) | 0·94 |

Inclusion of baseline ART status or AMBITION-cm treatment group did not alter the model findings. We assessed for multicollinearity among predictors using variance inflation factors , with a threshold of 10 indicating potential concern. The variance inflation factors for all predictors were below 1·7, indicating no significant multicollinearity. Goodness-of-fit was assessed using the Hosmer–Lemeshow test. The Hosmer–Lemeshow test indicated acceptable model fit (p>0·05 for each). The unadjusted EBV CNS models included 708 complete cases records. The adjusted EBV CNS models included 678 complete cases records. aOR=adjusted odds ratio. CSF=cerebrospinal fluid. cOR=crude unadjusted odds ratio. EBV=Epstein–Barr virus. *Adjusted for age (continuous variable), baseline CD4 cell count (continuous variable), sex, and study site.

***Table 5:*** **Multivariate analysis of the association between EBV co-infections and mortality at 2 and 10 weeks using logistic regression modelling**

## Discussion

In our cohort of 811 adults with advanced HIV disease and cryptococcal meningitis recruited from five African countries, we demonstrate that EBV and cytomegalovirus co-infections were very common. Approximately half of participants had evidence of cytomegalovirus viraemia, and 5% had cytomegalovirus CNS co-infection; 73% had EBV viraemia, and 27% had EBV CNS co-infection. Although co-infection with each of these ubiquitous herpes viruses was common in this severely immunocompromised cohort, the two viruses were associated with contrasting clinical phenotypes. EBV co-infections were associated with higher median CD4 cell counts and a proinflammatory cryptococcal profile known to be associated with improved survival and were not associated with poor outcomes. Conversely, cytomegalovirus viraemia and CNS co-infection were associated with a pauci-inflammatory response, and higher fungal burdens. High-level cytomegalovirus viraemia (≥1000 copies per mL) was significantly and independently associated with greater than double the odds of mortality at 2 weeks and 10 weeks, regardless of adjustment for potential confounders; cytomegalovirus CNS co-infection was not associated with higher mortality. Cytomegalovirus viraemia represents a potentially modifiable risk factor to improve survival among adults with cryptococcal meningitis.

Our prevalence data demonstrating a high proportion of both plasma and CSF EBV and cytomegalovirus co-infections among adults with HIV-associated cryptococcal meningitis are consistent with previous data from Africa.[9,10,12,18] Our finding that cytomegalovirus viraemia is associated with increased mortality is also concordant with earlier data from Uganda; in a cohort of 111 adults with HIV-associated cryptococcal meningitis, cytomegalovirus viraemia was associated with a three-fold increased hazard of 10-week mortality despite multivariate adjustment.[7] The notably larger sample size of our current study, including 811 adults from five countries, provides greater precision and generalisability to our estimates. Our data demonstrate that while cytomegalovirus viraemia was associated with an increased 2-week and 10-week mortality, the increased mortality risk was driven by participants with high-level cytomegalovirus viraemia. Risk of mortality at 2 and 10 weeks in participants with cytomegalovirus viraemia of 1000 copies per mL or greater was double that of participants with no or low-level cytomegalovirus viraemia, suggesting a dose–response relationship. These are the first published data to investigate the prognostic significance of cytomegalovirus CNS co-infection in the context of HIV-associated cryptococcal meningitis. Although the increased mortality risk among participants with cytomegalovirus CNS co-infection was not significant (likely due to the small sample size) the consistent direction and magnitude of the observed difference adds to our understanding of cytomegalovirus as a potentially significant copathogen in this setting.

Whether the associations between cytomegalovirus infection and poor outcomes in the context of HIV represents a causal relationship is unknown. Considering that both HIV and cryptococcosis are associated with marked immune dysregulation that might trigger cytomegalovirus reactivation with transient viraemia or CSF positivity, it is possible that in the context of advanced HIV disease, cytomegalovirus co-infections could be non-pathogenic markers of immunosuppression—a so-called bystander infection.[19] However, the indirect effects of cytomegalovirus reactivation and the downstream immunomodulatory effects of cytomegalovirus are now well recognised. Cytomegalovirus reactivation is highly immunogenic and is associated with cytomegalovirus-induced T-cell differentiation and upregulation of type 1 interferons, such as IFN-α. Type 1 interferons have important antiviral activity but downregulate type 2 interferons, such as IFN-γ produced by type 1 T-helper cells responding against intracellular pathogens including *Cryptococcus* and tuberculosis. In theory, this downregulation might increase disease severity of these opportunistic infections.[20] Our data demonstrating a higher median CSF fungal burden in people with cytomegalovirus co-infections support this hypothesis; as do our earlier data from Uganda that demonstrated that among adults with HIV-associated cryptococcal meningitis, active high-level cytomegalovirus viraemia (≥1000 IU/mL) was associated with double the hazard of incident tuberculosis disease during follow-up.[21] In our current cohort, our finding that participants with cytomegalovirus viraemia had a pauci-inflammatory cryptococcal phenotype characterised by lower peripheral CD4 cell counts, and lower CSF white cell counts and CSF protein measurements, indicates a potential additive immunodeficiency driven by cytomegalovirus. Human interleukin-10 (IL-10) is an immunomodulatory anti-inflammatory cytokine, which suppresses and controls the magnitude of the host response to avoid excessive immune activation.[22] In active cytomegalovirus infection, cytomegalovirus viral IL-10 is produced, and as a functional homologue of human IL-10, cytomegalovirus viral IL-10 competes with human IL-10 binding sites and acts as a super-agonist that limits inflammation with more potency than human IL-10.[22] In this manner, we speculate that cytomegalovirus reactivation in the context of advanced HIV disease might further compromise the immune response driving worse outcomes, and further investigation is warranted in this exploratory field.

In contrast to the potentially copathogenic role of cytomegalovirus, EBV infections within our cohort were not associated with adverse outcomes. This finding is consistent with our earlier data from Botswana demonstrating that among a cohort of 66 participants with cryptococcal meningitis, EBV CNS co-infection was associated with reduced in-hospital mortality.[23] In our current cohort, in contrast to cytomegalovirus viraemia, which was associated with less inflammation and increased mortality, EBV co-infections were associated with higher CD4 cell counts, and increased inflammation. EBV CNS co-infection was, in particular, associated with markedly more CSF inflammation with a positive association evident between EBV co-infection and CSF pleocytosis. Although it might be hypothesised that EBV is causing the additive inflammatory response due to invasion of the brain parenchyma and concurrent EBV meningoencephalitis, if this was the case it is expected the detection of EBV in the CSF would be associated with increased mortality within the cohort, which was not the case. Therefore, we propose that the observed association between EBV detection and inflammation, taken together with the survival analyses, suggest that in context of HIV-associated cryptococcal meningitis, EBV co-infections should be regarded as bystander infections indicative of a heightened host-mediated immune response, which is known to be protective in cryptococcal meningitis.[24]

Our study has several strengths. This is the largest study to date that characterises the epidemiology of cytomegalovirus and EBV plasma and CNS co-infections among adults with HIV-associated cryptococcal meningitis, and our large sample size of 811 participants ensures good precision of our prevalence and mortality estimates. Baseline and clinical outcome data were comprehensively collected as part of a multi-site phase 3 randomised controlled trial with no participants lost to

follow-up. Participants were recruited from seven sites, in five African countries, which suggests our data have good generalisability to the settings in which the majority of patients with cryptococcal meningitis are cared for globally. Our study had several limitations. First, cytomegalovirus end-organ disease was not systematically screened for, and therefore while we are able to report on associations with co-infection status and mortality, we are not able to stratify these estimates by end-organ disease status. Second, although our EBV and cytomegalovirus assay had a low limit of detection, due to the inherent limitations of qPCR assays it is possible that some very low-level EBV or cytomegalovirus co-infection cases could have been misclassified as cases with no co-infection. Third, considering that we were investigating four discrete exposure variables within our study, we recognise the risk for type 1 statistical errors due to multiple testing. Finally, we cannot discount the existence of unmeasured confounders within our analysis (eg, additional CNS herpes virus infections[25]) given the context of advanced HIV disease and the uncertainty with the causal pathways underpinning these analyses.

In conclusion, there is a growing body of evidence that despite ART, cytomegalovirus co-infections are associated with worse outcomes in the context of advanced HIV disease. These data contrast with survival associations observed with EBV co-infections within our cohort. Cytomegalovirus viraemia represents a potentially modifiable risk factor to improve survival among adults with advanced HIV disease and cryptococcal meningitis. To advance this field, we call for improved access to cytomegalovirus viral load testing facilities in the resource-limited settings where the majority of patients with advanced HIV disease are cared for globally, and highlight the potential benefits that would be afforded by a point-of-care cytomegalovirus quantitative lateral flow assay. Interventional trials are now required and planned to understand how treatment of cytomegalovirus viraemia impacts immunomodulation and clinical outcomes in advanced HIV disease.

#### Contributors

JE, EG, RD, DM, DB, TSH, and JNJ contributed to the conceptualisation and methodology of the study, and acquisition of funding. EG, RD, TSH, and JNJ provided supervision. JE, EG, RD, DL, DM, DB, HCM, CK, MCH, GM, CM, MM, CEN, TSH, and JNJ contributed to data curation, investigation, and project administration. JE and JNJ led on formal analysis and data visualisation. JE wrote the initial draft of the manuscript. JE and JNJ accessed and verified the data. All authors reviewed and contributed to the final draft of the paper. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

#### Declaration of interests

We declare no competing interests.

#### Data sharing

Data collected as part of this study will be made available to other on reasonable request to the AMBITION-cm trial management group. A dataset containing all data required to reproduce the analyses reported in the paper and supplementary appendix will be uploaded to the London School of Hygiene & Tropical Medicine data repository where it will be freely available at https://datacompass.lshtm.ac.uk/.

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