



# Cytomegalovirus infection is associated with increased mortality in the older population

George M. Savva,<sup>1\*</sup> Annette Pachnio,<sup>2</sup> Baksho Kaul,<sup>2</sup> Kevin Morgan,<sup>3</sup> Felicia A. Huppert,<sup>4</sup> Carol Brayne,<sup>1</sup> Paul A. H. Moss<sup>2</sup> and on behalf of the Medical Research Council Cognitive Function and Ageing Study

<sup>1</sup>Department of Public Health and Primary Care, Institute of Public Health, University of Cambridge, Robinson Way, Cambridge, CB20SR, UK

<sup>2</sup>School for Cancer Sciences, University of Birmingham, Birmingham, B15 2TT, UK

<sup>3</sup>School of Sport, Exercise and Health Sciences, Loughborough University, Ashby Road, Loughborough, UK

<sup>4</sup>Department of Psychiatry, Addenbrookes Hospital, University of Cambridge, Cambridge, UK

## Summary

**Cytomegalovirus (CMV) is a common herpesvirus infection and stimulates the expansion of very large numbers of CMV-specific T cells that reduce the CD4/CD8 ratio and suppress the number of naïve T cells. CMV infection has been associated with frailty and impaired survival. We investigated the correlates of CMV and the impact of the CMV infection on mortality within a cohort of 511 individuals aged at least 65 years who were followed up for 18 years. The mean age of the participants was 74 years of which 70% were CMV-seropositive. CMV was strongly linked to socio-economic status, and CMV infection increased the annual mortality rate by 42% (Hazard ratio = 1.42, 95% CI: 1.11–1.76 after adjusting for age, sex and baseline socio-economic and health variables) corresponding to 3.7 years lower life expectancy from age 65. Infection was associated with a near doubling of cardiovascular deaths, whereas there was no increase in mortality from other causes. These results show that CMV infection markedly increases the mortality rate in healthy older individuals due to an excess of vascular deaths. These findings may have significant implications for the study of immune senescence and if confirmed more generally could have important implications for measures to optimize the health of the elderly.**

**Key words:** cytomegalovirus; elderly; mortality; cardiovascular.

## Introduction

Cytomegalovirus (CMV) is a member of the human herpesvirus family and is widely prevalent in the population (Crough & Khanna,

2009). In common with all herpes viruses, CMV is not cleared from the host after infection but viral replication is suppressed by immune surveillance mediated by the humoral and cellular immune systems. CMV is arguably the most immunodominant of all infectious agents and leads to expansion of a large population of CMV-specific CD8+ T cells (Gillespie *et al.*, 2000). The CMV-specific CD4+ T-cell response is also substantial but less markedly expanded and infection therefore leads to a reduction in the CD4/CD8 ratio (Gratama *et al.*, 1987). In addition, the large increase in the T-cell memory pool leads to a decrease in the number of circulating naïve T cells and may induce a low-grade inflammatory state (Weinberger *et al.*, 2007). The clinical importance of CMV in patients who are immune suppressed is widely appreciated and the virus is a significant cause of morbidity and mortality in settings such as allogeneic transplantation or HIV infection. However, increasing attention is now being given to the potential clinical importance of CMV infection in the immune competent population. CMV seropositivity appears to carry particular significance for elderly donors and this is likely to relate to the magnitude of the CMV-specific immune response in this age group. In contrast to the immune response against many human pathogens, the T-cell immune response to CMV increases with age and can comprise up to 40% of circulating CD8+ T cells in elderly donors (Khan *et al.*, 2004). The reasons for this are unclear but may relate to episodes of subclinical viral reactivation, which serve to repeatedly boost the CMV-specific immune repertoire.

Cytomegalovirus infection is strongly associated with the development of frailty in the older population, most notably in individuals with high CRP levels as a marker of underlying inflammation (Schmaltz *et al.*, 2005). Studies in cohorts of individuals aged over 80 or 90 years have associated CMV seropositivity with an 'immune risk phenotype' that is a marker of adverse outcomes including reduced survival (Wikby *et al.*, 2002). Two studies have linked high CMV antibody titres with mortality in elderly cohorts studied for up to 7 years and a recent study found a relationship with all-cause mortality among a large sample representative of the US population aged 25 and over (Strandberg *et al.*, 2009; Wang *et al.*, 2010; Simanek *et al.*, 2011). The mechanism underlying this relationship is not known, and associations with respect to cause-specific mortality have been mixed.

There is a substantial amount of literature that links CMV infection to an increased incidence of cardiovascular disease. The HOPE study revealed a relative risk of 1.24 for atherosclerosis in CMV-seropositive individuals (Smieja *et al.*, 2003), although this association has not been seen in all studies (Haider *et al.*, 2002). Simanek recently analysed the NHANES III database to show that CMV infection was associated with an increase in cardiovascular disease in those with high CRP levels (Simanek *et al.*, 2011). CMV infection is most clearly associated with vascular disease following cardiac transplantation where antiviral drugs have been shown to

## Correspondence

Paul Moss, School for Cancer Sciences, University of Birmingham, Birmingham, B15 2TT, UK. Tel.: +44-121 414 2824; fax: +44-121 414 4486; e-mail: p.moss@bham.ac.uk

\*Present address: The Irish Longitudinal Study on Ageing, Trinity College Dublin, Dublin 2, Ireland.

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limit the degree of tissue damage (Potena *et al.*, 2006). An association between CMV infection and other major causes of mortality is less well established, although the virus has been suggested a potential aetiological agent in the development of glioma (Lucas *et al.*, 2011).

Epstein-Barr Virus (EBV) is also a ubiquitous herpes virus and is associated with a range of clinical complications. The T-cell immune response to EBV is substantial and the EBV-specific immune response also increases with age (Glaser *et al.*, 1985), although there have been no studies to address the potential importance of EBV infection on survival in the elderly.

Here, we present an analysis of the effect of CMV and EBV infection status on overall and cause-specific mortality over an 18-year follow-up in a cohort of 511 individuals aged 65 years and over that were randomly selected from the general older population. These data are used to estimate life expectancy in this population from age 65 in relation to CMV status. This reveals that CMV-seropositive individuals at age 65 have a median life expectancy nearly 4 years shorter than uninfected individuals.

## Results

### Baseline correlates of CMV and EBV serostatus

The age of subjects at entry in the study ranged from 65 to 94 years with a mean of 74.1 years (standard deviation, 6.0 years) and 51% of subjects were women. The estimated prevalence of CMV positivity in the population is 70% (95% CI: 65–74) and seroprevalence did not vary in relation to gender ( $P = 0.36$ ) or age ( $P = 0.48$ ).

The Healthy Aging Study excluded the severely disabled, but among the remaining sample, there was a relationship between CMV positivity and disability. Of those without disability, 67% were CMV-seropositive compared to 79% of those with instrumental activity of daily living (IADL) impairment and 86% of those with an activity of daily living (ADL) impairment ( $P = 0.047$ ). There was no association between CMV seroprevalence and the number of comorbid conditions ( $P = 0.88$ ) or self-rated health ( $P = 0.26$ ), and none of the comorbid conditions examined were significantly associated with CMV seropositivity (data not shown), although there was some evidence that those with angina were more likely to be CMV-positive (56/72 = 77%) than those without (267/399 = 68%,  $P = 0.16$ ). CMV positivity was higher in those who left school without qualifications (74%) compared with those who achieved some qualifications (61%,  $P = 0.009$ ) and was higher in subjects in lower social classes (Table 1,  $P = 0.004$ ).

In contrast to CMV, no relationship was observed between socio-demographic or health-related factors and EBV serostatus (Table 2). However, EBV infection was positively correlated with CMV serostatus. Ninety-one per cent of CMV-seropositive individuals were also EBV-seropositive compared with only 82% of those in the CMV-seronegative group. (OR = 2.2, CI: 1.2–4.1,  $P = 0.006$ ; Supplementary Fig. S1). This association is not affected by adjusting for social class, area-level deprivation, education or health-related factors (multivariate OR = 2.2, CI: 1.2–4.0,  $P = 0.012$ ) and so

**Table 1** The prevalence and correlates of Cytomegalovirus (CMV) seropositivity

	CMV-Negative N (%) <sup>*</sup>	CMV-Positive N (%) <sup>*</sup>	% CMV -positive <sup>*</sup>	
Total	148	323	70	
Did not die	55 (42)	73 (23)	55	
Died	93 (58)	250 (77)	75	$P = 0.001$
Male	75 (45)	154 (40)	67	
Female	73 (55)	169 (60)	71	$P = 0.36$
Age (Years)				
65–69	44 (36)	89 (30)	66	
70–74	42 (31)	81 (29)	68	
75–79	32 (17)	84 (24)	76	
80–84	22 (12)	53 (14)	72	
85+	8 (4)	16 (4)	69	$P = 0.48$
Never smoked	46 (30)	107 (35)	72	
Previous smoker	76 (49)	164 (48)	69	
Current smoker	26 (20)	52 (17)	66	$P = 0.60$
Not disabled	130 (89)	267 (80)	67	
IADL disabled	15 (9)	41 (15)	79	
ADL disabled	3 (2)	15 (5)	86	$P = 0.047$
0 co-morbid conditions	32 (22)	79 (24)	71	
1	57 (38)	112 (35)	68	
2	41 (28)	84 (24)	66	
3+	18 (12)	48 (16)	76	$P = 0.55$
Excellent self-rated health (SRH)	39 (27)	66 (21)	64	
Good SRH	80 (52)	172 (53)	70	
Fair/Poor SRH	28 (20)	85 (26)	75	$P = 0.26$
No qualifications	82 (57)	217 (70)	74	
Qualified	66 (43)	106 (30)	61	$P = 0.009$
Social class				
Missing	1 (1)	0 (0)	0	
I	5 (3)	4 (1)	45	
II	42 (28)	63 (17)	59	
IIIN	27 (17)	41 (13)	63	
IIIM	55 (39)	161 (51)	75	
IV	17 (11)	35 (11)	70	
V	1 (0)	15 (5)	96	
(Exempt)	0 (0)	4 (1)	100	$P = 0.004$
Least deprived quartile	42 (28)	71 (20)	62	
2 <sup>nd</sup>	38 (27)	78 (25)	68	
3 <sup>rd</sup>	35 (26)	83 (29)	72	
Most deprived quartile	29 (19)	82 (24)	75	$P = 0.24$

IADL, instrumental activity of daily living; ADL, activity of daily living.

<sup>\*</sup>All prevalences (%) are back-weighted to the population.

indicates shared susceptibility to co-infection as opposed to common exposures.

### The influence of CMV and EBV serostatus on overall and cause-specific mortality

A total of 378 participants had died at the census point of 31 December 2008. Fig. 1 shows the effect of CMV seropositivity on survival as measured in years from age 65. CMV seropositivity was associated with a 40% increase in the rate of mortality (HR = 1.40, 95% CI: 1.10–1.78). This value is not substantially attenuated by adjusting for date of birth and sex (HR = 1.47, CI: 1.16–1.87), nor

**Table 2** The prevalence and correlates of Epstein–Barr Virus (EBV) seropositivity

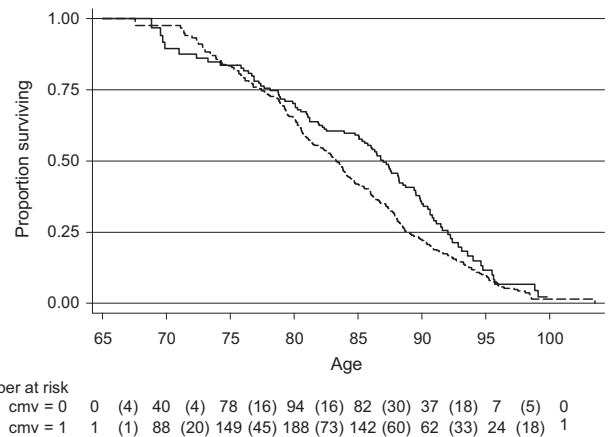
	EBV-Negative N (%)*	EBV-Positive N (%)*	% EBV -positive*	
Total	54	435	89	
Did not die	17 (33)	112 (28)	87	
Died	37 (66)	323 (72)	90	$P = 0.37$
Male	28 (42)	211 (42)	89	
Female	26 (58)	224 (57)	89	$P = 0.64$
Age (years)				
65–69	16 (33)	121 (31)	89	
70–74	10 (21)	122 (32)	93	
75–79	14 (19)	106 (22)	91	
80–84	8 (19)	69 (13)	84	
85+	6 (8)	17 (4)	77	$P = 0.20$
Never smoked	20 (40)	140 (33)	87	
Previous smoker	23 (37)	224 (49)	92	
Current smoker	11 (22)	71 (18)	87	$P = 0.46$
Not disabled	48 (81)	363 (83)	90	
IADL disabled	6 (19)	54 (13)	85	
ADL disabled	0 (0)	18 (4)	100	$P = 0.29$
0 comorbid conditions	14 (24)	97 (23)	89	
1	21 (34)	155 (38)	90	
2	10 (18)	126 (27)	93	
3+	9 (25)	58 (13)	82	$P = 0.24$
Excellent self-rated health (SRH)	15 (26)	90 (22)	88	
Good SRH	27 (48)	238 (54)	90	
Fair/Poor SRH	12 (26)	107 (24)	89	$P = 0.75$
No qualifications	32 (63)	285 (68)	90	
Qualified	22 (37)	150 (32)	88	$P = 0.52$
Social class				
Missing	0 (0)	1 (0)	100	
I	1 (1)	9 (2)	90	
II	15 (23)	88 (20)	87	
IIIN	8 (12)	69 (16)	90	
IIIM	23 (48)	196 (46)	89	
IV	7 (13)	46 (12)	89	
V	0 (0)	18 (4)	100	
(Exempt)	0 (0)	5 (1)	100	$P = 0.91$
Least deprived quartile	10 (17)	108 (23)	92	
2 <sup>nd</sup>	13 (22)	106 (26)	91	
3 <sup>rd</sup>	15 (35)	109 (28)	87	
Most deprived quartile	15 (26)	101 (23)	88	$P = 0.63$

IADL, instrumental activity of daily living; ADL, activity of daily living.

\*All prevalences (%) are back-weighted to the population.

by adjusting for social class, area-level deprivation, education or smoking ( $HR = 1.42$ ,  $CI: 1.11–1.82$ ), nor when additionally including potential mediators of the effect of CMV, the presence of comorbid conditions, disabilities or self-rated health (full multivariate model  $HR = 1.35$ ,  $CI: 1.04–1.75$ ).

Figure 1 shows the Kaplan–Meier curves of survival from age 65 by CMV serostatus. While it appears that CMV-seronegative participants had a higher mortality rate between the ages of 65 and 70, very few participants were enrolled in the study between these ages and only five participants died in this age range. After the age of 70, the effect of CMV seropositivity on mortality is apparent.



**Fig. 1** Kaplan–Meier survival curves showing the effect of Cytomegalovirus (CMV) seropositivity on survival from age 65 among the seropositive (dashed line) and seronegative (solid line) populations. The table below shows the number at risk at the start of each time point, and the number of deaths between time points is shown in brackets. There are few people ‘at risk’ between the ages of 65–70, and so survival estimates in this time period are unreliable. More participants are under observation between the ages of 70 and 90 where the curve clearly shows worse survival in the CMV-positive group.

**Table 3** The median (25<sup>th</sup> and 75<sup>th</sup> percentiles in brackets) of life expectancy from 65<sup>th</sup> birthday, according to sex and Cytomegalovirus (CMV) seropositivity

	75% surviving (years)	Median survival time (years)	25% surviving (years)
Men			
CMV+	10.3 (8.0–12.8)	15.8 (14.0–18.5)	22.2 (19.8–23.6)
CMV–	11.1 (4.5–14.9)	20.1 (11.1–23.7)	25.9 (22.5–29.0)
Total	10.9 (8.0–12.7)	16.4 (14.5–18.9)	23.2 (21.6–24.9)
Women			
CMV+	14.4 (10.7–16.1)	20.3 (17.6–22.4)	27.0 (24.7–29.2)
CMV–	15.5 (3.8–21.5)	23.2 (20.5–25.5)	27.9 (25.0–30.7)
Total	15.0 (11.2–16.2)	21.8 (18.9–23.1)	27.4 (25.5–29.2)

Overall, median life expectancy from age 65 is 22.0 years ( $CI: 17.4–23.7$ ) in those without CMV infection and 18.3 years ( $CI: 16.1–19.4$ ) in subjects who are CMV-seropositive (log rank test  $P = 0.005$ ). The 75<sup>th</sup> percentile, median and 25<sup>th</sup> percentile of survival times for men and women in this group from age 65 is shown in Table 3.

We next explored the link between CMV seropositivity and mortality from specific causes within our the cohort. Cardiovascular death was found to be markedly more common in the CMV-seropositive group (Table 4), and the subhazard ratio for cardiovascular death is 1.95 (95%  $CI: 1.29–2.96$ ,  $P = 0.002$ ). Interestingly, there is no statistically significant effect of CMV on any other cause of death (subhazard ratio for all noncardiovascular deaths combined: 1.00 95%  $CI: 0.74–1.33$ ).

After adjusting for age and sex, there was some evidence of an effect of EBV on survival, although this was not statistically significant ( $HR = 1.28$ ,  $CI: 0.91–21.8$ ,  $P = 0.157$ ) possibly due to the small number of individuals in the sample without EBV making any effect of EBV difficult to detect. The effect of CMV or EBV on

**Table 4** The distribution of causes of death by Cytomegalovirus (CMV) seropositivity and subhazard ratios for the effect of CMV on cause-specific mortality. Subhazard ratio estimated using competing risks regression adjusted for date of birth and sex

Cause of death	CMV-negative N (% of deaths)	CMV-positive N (% of deaths)	Subhazard ratio (95% CI)
Cardiovascular (ICD10 = I00-I99)	28 (30)	110 (44)	1.94 (1.29–2.93)
Cancer (ICD10 = C00-D48)	30 (33)	57 (21)	0.90 (0.58–1.40)
Respiratory (ICD10 = J00-J99)	17 (18)	44 (16)	1.21 (0.69–2.11)
Other	18 (20)	39 (19)	0.86 (0.50–1.49)
Total	93	250	

mortality was not altered by adjusting for the other virus, and dual positivity was also not associated with any increased risk (HR for interaction = 1.20, CI: 0.58–2.51,  $P = 0.620$ ). None of the results were substantially different if time since cohort entry instead of age was used as the timescale in the survival analysis.

## Discussion

Cytomegalovirus is acknowledged as an important pathogen in the setting of immune suppression but relatively little attention has been given to its potential effects on the health of the general population. Our study reveals that CMV infection has a strong influence on overall survival in a cohort aged 65 years and over, such that those participants who were CMV-seropositive had a median life expectancy that was 3.7 years shorter. In contrast, infection with Epstein–Barr virus had no statistically significant effect on survival, although small numbers without EBV infection made its socio-economic correlates and effect on mortality difficult to detect.

Complete notification of mortality was available over an 18-year follow-up period during which time 74% of participants died. Seventy per cent of subjects were CMV-seropositive in this cohort that is typical of infection rates at this age within Northern Europe and USA (Cannon *et al.*, 2010). CMV infection rates vary markedly in different populations with seropositivity approaching 100% in many parts of the world and our findings indicate the potential value of similar studies in additional cohorts. The life expectancy estimates in this subsample of the MRC Cognitive Function and Aging Study are in line with those from the complete cohort (Jagger *et al.*, 2007). This subsample was recruited on the basis that participants showed no evidence of severe mental or physical disability at the time of entry, but it is unlikely that this selection could account for the associations we have described. Our analysis assumed that CMV seropositivity would not change during follow-up and this is likely to be a reasonable assumption for the majority of individuals in this age group. We included three measures of socio-economic status in our study: educational attainment, social class defined by occupation and area-level deprivation. Adjusting for these potential socio-economic confounders only very slightly attenuated our estimates for the risk of CMV at all, so while it is

possible that our results reflect residual confounding with true socio-economic status, we feel this is unlikely.

Although the proportional hazards assumption was not statistically and significantly violated, the effect of CMV seropositivity on mortality that we report appeared to be reversed between the ages of 65 and 70. However, very few participants were entered into the study before the age of 70, and the initial selection criteria of being without severe physical or cognitive impairment may have introduced a bias early in the follow-up period whereby those who are CMV-seropositive yet still without these impairments have better immunological control over their infection. This would lead to an apparently higher mortality rate in the CMV-seronegative participants soon after study selection.

The MRC CFAS cohort from which our sample was recruited is from a single urban centre; however, our sample is relatively small and homogenous and so our results must be replicated in larger more diverse cohorts before they can be reliably generalized. Nevertheless, these findings are supported by a number of studies which have suggested that the immune response to CMV is an important determinant of health in the elderly. The OCTO and NONA studies of donors aged over 80 years have revealed that CMV infection is associated with an ‘immune risk phenotype’ that has been shown to be correlated with death. More recently, the antibody titre against CMV is emerging as an important correlate of health. Three recent studies in subgroups of the older population have demonstrated an effect of CMV on mortality. Strandberg *et al.* (Strandberg *et al.*, 2009) showed an increase in 7-year mortality risk with increasing CMV-specific antibody titre in a sample of 383 participants with stable cardiovascular disease aged 75–90. Wang *et al.* (Wang *et al.*, 2010) showed a similar relationship in a cohort of 635 women from Baltimore over a 5-year follow-up period. Most recently, Simanek *et al.* (Simanek *et al.*, 2011) found an association between CMV seropositivity and all-cause mortality in a large sample of US adults aged between 25 and 90, consistent with our findings. A number of possible mechanisms by which CMV infection may increase mortality risk are now emerging. These relate primarily to the very large CMV-specific immune response that develops after infection and which can expand further in elderly donors (Vescovini *et al.*, 2007). The consequence of the accumulation of large numbers of memory T cells in blood is that the CD4/CD8 ratio and the number of naïve T cells are both reduced (Chidrawar *et al.*, 2009). Indeed, an inverted CD4/CD8 ratio has already been shown to be a marker of increased mortality rate within the Cambridge-shire cohort of the healthy aging study (HR = 1.36) (Huppert *et al.*, 2003). CMV-seropositive individuals may thus exhibit increased susceptibility to infection and the association between CMV infection and poor immune response to influenza vaccination is provocative in this regard (Trzonkowski *et al.*, 2009).

Our data are the first to specifically address the role of CMV infection on mortality on a large homogeneous cohort of healthy elderly donors with extensive follow-up such that mortality was reached in over three quarters of the cohort. We have shown that that CMV seropositivity is strongly associated with an increased risk of death from cardiovascular disease where the death rate from these complications is nearly doubled while deaths from other



causes are unaffected. Strandberg *et al.* (Strandberg *et al.*, 2009) discovered no significant difference in causes of death between those in the highest and lowest CMV quantiles, although only 33 and 46 deaths occurred in these groups, respectively. Simanek *et al.* did not find that CMV infection was associated with an increase in the risk of CVD mortality in the NHANES study, although their population was much younger with a mean age of < 50 years (Simanek *et al.*, 2011). In support of our results, several other findings have also suggested that CMV seropositivity may confer a specific increase in cardiovascular mortality. CMV is associated with vascular disease in a range of clinical settings including cardiac allograft arteriopathy (Streblov *et al.*, 2008) and dialysis-associated atherosclerosis (Betjes *et al.*, 2010). Infection is also associated with impaired vascular function in young immunocompetent donors (Grahame-Clarke *et al.*, 2003) and these data indicate that increased attention should be given to the contribution of CMV infection in the development of vascular disease in the elderly. Donors with raised inflammatory markers have been reported as being at increased risk of clinical complications associated with CMV infection, although it was recently shown that the inflammation associated with aging ('inflammaging') is not itself directly linked to CMV infection (Bartlett *et al.*, 2012). Unfortunately, inflammatory markers were not available for study within this cohort.

Our data indicate that CMV infection is strongly associated with increased risk of cardiovascular death in people over the age of 65 years. As has been shown previously and confirmed in our study, CMV infection is strongly related to socio-economic status and infection may therefore be one potential mechanism by which inequalities in health and mortality can develop between socio-economic groups.

## Experimental procedures

### Study population

The ERSC Healthy Aging Study was designed to investigate the social and psychological determinants of successful aging using a subsample of the Medical Research Council Cognitive Function and Aging Study (MRC CFAS: <http://www.cfes.ac.uk>). MRC CFAS included 13 004 individuals representative of the population aged 65 and over from five centres around England and Wales. Participants were selected from lists of primary care physicians covering the populations in specific geographic areas that included institutions that cared for elderly people. Response rate was high. Stratified selection was used so that equal numbers of those aged 65–74 and 75 and over were sampled. Participants underwent an initial screening interview, including assessments of cognitive function, health and disability. All of those with cognitive impairment, as well as a stratified sample of the remainder, underwent a comprehensive cognitive and psychological assessment.

Participants from the Cambridgeshire and Nottingham centres not selected to the full assessment ( $n = 3133$ ) and who were without severe cognitive (MMSE  $\geq 18$  and AGE-CAT organicity level < 03) or physical ( $\geq 8$  on Clackmannan ADL scale) impairment ( $n = 2365$ ) were eligible for inclusion in the ESRC Healthy Aging

Study (Huppert *et al.*, 2003). Of these 2365 individuals without severe cognitive impairment or disability, 292 refused to participate and 32 could not be contacted, resulting in a total of 2041 participants interviewed in Cambridgeshire and Nottingham.

Of 1020 respondents in the Nottingham sample, blood samples were provided by a randomly selected sample of 533, of which 511 were available for the present analysis.

Notification of mortality for all study participants was obtained from national records. Cause of death was coded into four groups by ICD-10 code as being caused by cancer (ICD-10 = C00–D48), cardiovascular disease (ICD-10 = I00–I99), respiratory disease (ICD-10 = J00–J99) or other causes.

### Determination of CMV and EBV seroprevalance

ELISA assays were used to determine the presence of antibodies against CMV or EBV viral antigens. The CMV serostatus was determined using an in-house ELISA detecting anti-CMV IgG antibodies. Briefly, CMV-lysate or mock-lysate was coated on to ELISA plates (Nunc Maxisorb) overnight. Plates were then washed with 1xPBS + 0.05% Tween20 and blocked with 1xPBS + 1% BSA + 0.05% Tween20. 100 mL of a 1:400 dilution of the patient serum was then added to lysate and mock-lysate coated wells for each serum to be tested accordingly. A mix of three healthy CMV-seropositive serum samples served as a standard curve. Following 1 h incubation at room temperature, plates were washed and an HRP-conjugated anti-human IgG antibody (1:8000; Southern Biotech, Birmingham, AL, USA) added for 1 h at room temperature. Plates were washed, and substrate solution (TMB; Rockland, Gilbertsville, PA, USA) was added and incubated for 10 min before stopping the colour development by the addition of 1M HCl and measured at 450 nm. For interpretation of the results background, OD against mock-lysate was deducted from OD read against CMV-lysate. Values of a corrected OD above 0.5 were interpreted as CMV-seropositive.

Epstein–Barr Virus serostatus was determined using a commercial EBV VCA IgG ELISA kit (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) according to manufacturer's instructions. Samples were classed as positive when  $> 12 \text{ U mL}^{-1}$  were detected, 8–12  $\text{U mL}^{-1}$  were classed as equivocal, and titres  $< 8$  were classed as negative.

A total of 323 samples were determined as CMV-seropositive, whereas 148 were CMV-seronegative. A total of 434 samples were EBV-seropositive, whereas 55 were EBV-seronegative.

Owing to poor quality samples, CMV serotyping was inconclusive in 40 participants, 35 of whom died before the censoring date. The ELISA assay for EBV was inconclusive in 22 cases, 18 of whom died. Missing serostatus was not linked to any socio-demographic or health-related covariate. Neither those with missing CMV status (HR = 1.2 CI: 0.86–1.7) nor those with missing EBV status (HR = 1.05 CI: 0.65–1.70) had a significantly higher mortality risk.

### Assessment of covariates

Age was included as a continuous variable or split into five groups (65–69, 70–74, 75–79, 80–84, 85+) as appropriate. Smoking was

self-reported as current smoker, ever smoked or never smoked. The presence or history of nine health conditions (heart attack, angina, stroke/transient ischaemic attack, intermittent claudication, diabetes, depression, arthritis, peptic ulcer, and high blood pressure) was recorded for each participant, and for descriptive analysis the number of these grouped into four groups (0, 1, 2, 3 or more). Self-rated health was assessed by the question 'Would you say that for someone of your age, your own health in general is:' with the possible responses 'excellent', 'good', 'fair' or 'poor'. Whether each participant was impaired in an IADL or an ADL was recorded. IADLs included being able to catch a bus, conduct heavy housework, carry heavy shopping bags or cook a meal. ADLs included cutting nails, climbing a flight of stairs, dressing, bathing and using the toilet. Level of education achieved was measured by asking the participant whether they had passed any examinations before they left school (yes or no). Among this cohort, having no qualifications corresponds closely to having completed 9 or fewer years of education. Social class was based on the highest occupation achieved during working life, or that of a husband in the case of housewives and was grouped into I and II including those with professional and managerial, IIIN-skilled nonmanual, IIIM-skilled manual, IV partly skilled and V-unskilled occupations.

Area-level deprivation was measured using the Townsend deprivation index, independently of individual socio-economic status. The Townsend index is a composite measure that takes into account the proportion unemployed in a geographic region (each region containing approximately 200 households), the proportion of households who do not possess a car, the proportion of households with more than one person per room, and the proportion of households that are not owner-occupied. The higher the score, the more deprived the area. We divided our sample into four roughly equally sized groups based on this score.

### Statistical analysis

The Healthy Aging Study was recruited by stratified sampling, and so a weight was applied to all prevalence estimates to ensure that these are applicable to the healthy older population. The weight used was an inverse probability weight based on the probability of inclusion in the study given a participant's age, sex and cognitive function at baseline. Correlates of CMV and EBV were assessed using chi-squared tests for statistical significance. The relationship between CMV and EBV seropositivity was assessed using logistic regression.

A Kaplan–Meier survival curve was used to illustrate the effect of CMV on survival and to calculate life expectancy in men and women from the age of 65 given CMV status. Cox proportional hazards regression was used to estimate the hazard ratio (along with 95% confidence intervals) associated with CMV seropositivity and EBV and to adjust for covariates. The joint effect of CMV and EBV was estimated by adding an interaction term into the model. Potential confounders of the relationship between CMV seropositivity and death were entered into the Cox survival model as covariates, and these included date of birth and sex, socio-economic status (educational attainment, social class, area-level deprivation) and

smoking status as a marker of health behaviour. Possible mediators of the relationship between seropositivity and mortality (chronic conditions entered individually into regression models, disability and self-rated health) were also included. See above for detail of how these covariates were assessed.

Left-censored survival analysis was used throughout with participants' age as the timescale. Subjects entered and were considered 'at risk' from the age at which they were recruited to the study until their age at death or their age at the censoring date of 31 December 2008. Age was used as the primary timescale for analysis owing to the wide age range of the sample at baseline making adjustment for age in a conventional survival analysis difficult. (Thiébaud & Bénichou, 2004; Cologne *et al.*, 2012). However, as all covariates, as well as EBV and CMV status, were measured at study entry and participants were required to be free of severe cognitive or physical impairment at study entry, a sensitivity analysis was conducted using time since study entry as the timescale.

The proportional hazards assumption was tested for all factors included in regression models using Schoenfeld residuals along with visual inspection of log-log plots and Kaplan–Meier curves.

Competing risks regression was used to estimate the effect of virus seropositivity on specific causes of death (Fine & Gray, 1999), these are presented as subhazard ratios for each cause of death. All analyses were conducted using STATA 12.0.

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### Authors' contributions

GS conducted the statistical analysis and contributed to manuscript. AP and BK performed laboratory analysis of CMV and EBV status. KM, FH and CB set up the CFAS study, organized sample collection. PM developed the collaboration and wrote the first draft of the manuscript.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1** A positive correlation is seen between the incidence of infection with Cytomegalovirus and Epstein-Barr Virus.