ARTICLE

Cytomegalovirus antibodies are associated with mood disorders, suicide, markers of neuroinflammation, and microglia activation in postmortem brain samples

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Cytomegalovirus (CMV) is a common, neurotrophic herpesvirus that can be reactivated by inflammation and cause central nervous system disease. We hypothesize that CMV may contribute to the neuroinflammation that underlies some psychiatric disorders by (1) exacerbating inflammation through the induction of anti-viral immune responses, and (2) translating peripheral inflammation into neuroinflammation. We investigated whether the presence of anti-CMV antibodies in blood were associated with mental illness, suicide, neuroinflammation, and microglial density in the dorsolateral prefrontal cortex (DLPFC) in postmortem samples. Data ($n = 114$ with schizophrenia; $n = 78$ with bipolar disorder; $n = 87$ with depression; $n = 85$ controls) were obtained from the Stanley Medical Research Institute. DLPFC gene expression data from a subset of 82 samples were categorized into "high" ($n = 30$), and "low" ($n = 52$) inflammation groups based on a recursive two-step cluster analysis using expression data for four inflammation-related genes. Measurements of the ratio of non-ramified to ramified microglia, a proxy of microglial activation, were available for a subset of 49 samples. All analyses controlled for age, sex, and ethnicity, as well as postmortem interval, and pH for gene expression and microglial outcomes. CMV seropositivity significantly increased the odds of a mood disorder diagnosis (bipolar disorder: OR = 2.45; major depression: OR = 3.70) and among the psychiatric samples, of suicide (OR = 2.09). Samples in the upper tercile of anti-CMV antibody titers were more likely to be members of the "high" inflammation group (OR = 4.41, an effect driven by schizophrenia and bipolar disorder samples). CMV positive samples also showed an increased ratio of non-ramified to ramified microglia in layer I of the DLPFC (Cohen's d = 0.81) as well as a non-significant increase in this ratio for the DLPFC as a whole (d = 0.56). The results raise the possibility that the reactivation of CMV contributes to the neuroinflammation that underlies some cases of psychiatric disorders.

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INTRODUCTION

Cytomegalovirus (CMV) is a common herpesvirus that establishes lifelong latent infections, undergoes periodic reactivation, and may cause disease in vulnerable populations including neonates and the immunocompromised. CMV has attracted the attention of the psychiatric field because CMV (a) is neurotrophic and may cause neurological disease [1, 2], (b) it can be reactivated by psychological stress which is linked with the onset and exacerbation of many psychiatric disorders [3, 4], and (c) it can be reactivated by inflammation [4–6], which is linked with multiple psychiatric disorders including mood disorders and schizophrenia [7–11]. Importantly, lytic CMV replication can in turn worsen inflammation [12, 13], raising the possibility that poorly-controlled CMV infections may be one source of the systemic or neuroinflammation underlying the development of some cases of mental illness [14, 15].

Most of the evidence linking CMV infections to psychiatric disorders is epidemiological [16]. At least 20 studies have reported either a higher frequency of seropositivity to CMV in depressed samples versus controls or a positive correlation between anti-CMV IgG levels and depressive symptoms [16]. In addition, two prospective studies found that CMV seropositivity was associated with an increased risk of subsequent depression [17, 18]. Similarly, a large Swedish cohort study showed that children hospitalized with a CMV infection were 16.6 times more likely to develop a non-affective psychosis in the future [19] and several studies have linked schizophrenia with CMV infection [20].

We recently published some of the first work linking CMV seropositivity to neuroimaging abnormalities in the context of major depressive disorder (MDD). In up to three independent samples, we demonstrated that compared with CMV seronegative individuals with MDD, CMV positive individuals with MDD had widespread reductions in gray matter volume, decreased white matter integrity in a tract connecting the frontal and occipital lobes (inferior frontal occipital fasciculus), and reduced functional

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connectivity between hubs of the sensorimotor and salience networks [21–23]. Leboyer and colleagues had previously reported that CMV antibody levels were inversely associated with hippocampal volume in individuals with bipolar disorder (BD) and schizophrenia [24]. Similarly, Agartz and colleagues recently found that CMV-positive patients with bipolar or schizophrenia spectrum disorders had smaller dentate gyri [25] and reduced total cortical area as compared with CMV-negative patients [26]. However, whether CMV is playing a causal role in these neuroimaging abnormalities and if so, whether these findings relate to a heightened inflammatory process, is still unclear. At least in vitro, herpesvirus infections trigger the production of a range of cytokines and chemokines by glial cells [27], but to our knowledge the link between CMV infection and inflammation in the central nervous system (CNS) has never been tested in people with psychiatric disorders.

Here, we investigated in a postmortem sample whether CMV serostatus and serum antibody levels to CMV were associated with the odds of: 1) having a psychiatric disorder, 2) dying by suicide, 3) having higher neuroinflammation (based on a previously performed clustering analysis of immune-related gene expression in the dorsolateral prefrontal cortex [9]), and 4) showing increased microglia activation in the dorsolateral prefrontal cortex (i.e., an increased ratio of non-ramified to ramified microglia). We hypothesized that CMV seropositivity and/or higher CMV antibody levels would be associated with increased odds of 1) diagnosis with a psychiatric disorder, 2), suicide, 3) assignment to the "high" neuroinflammation group, and 4) an increased ratio of non-ramified to ramified microglia.

METHODS

Postmortem samples

The Stanley Medical Research Institute's (SMRI) brain tissue collection is described in detail elsewhere [28]. In short, a standard neuropathological examination was performed by a pathologist with screening for cardiovascular disease, hemorrhage, trauma, tumors, dementia, hypoxia, or other pathology. All medical and psychiatric records were obtained and reviewed by two senior psychiatrists. A psychiatrist also contacted one or more family members by telephone before making a final DSM-IV diagnosis. For the controls, a psychiatrist conducted a structured telephone interview with first-degree family members to obtain all pertinent psychiatric and medical history.

Trained medical examiners used a standardized protocol to collect and process brain tissue. The cerebrum was hemisected, with one hemisphere fixed in formalin and the other sliced into 1.5 cm thick coronal sections and frozen in a mixture of isopentane and dry ice. Formalin fixation and freezing were randomly alternated between the right and left hemispheres. The serum was from blood obtained from the heart at autopsy.

Of the 367 serum samples obtained from the SMRI's postmortem brain collection, we excluded one case with a primary diagnosis of alcohol dependence, one without diagnostic information, and one with indeterminate CMV serology results. Therefore 364 samples ($n = 114$ with schizophrenia; $n = 78$ with BD; $n = 87$ with a depressive disorder; $n = 85$ controls) were included in the statistical analyses. A total number of 119 samples died by suicide ($n = 34$ with schizophrenia; $n = 37$ with BD; $n = 48$ with depressive disorder). Demographic details and medical/medication information are shown in Table 1 and Supplementary Table S1 and Supplementary Table S2.

The Array Collection is a cohort of brain tissue samples, 35 schizophrenia cases, 35 bipolar disorder cases, and 35 matched controls, that was selected from the SMRI brain tissue collection in 2002. The purpose of the Array Collection was to provide researchers with brain tissue samples for high throughput gene expression, proteomic, and metabolomic studies. The diagnostic groups were well-matched for demographic features such as age, sex, and postmortem interval (PMI). Fresh frozen, 14um thick sections were cut from the DLPFC (BA46) of the Array Collection and made available (all 105 samples) to Dr. Weickert's laboratory for gene expression analyses. The results of the analyses comparing the inflammation-related gene expression of schizophrenia, bipolar disorder, and control samples from this cohort have been published previously [9].

A subset of 60 samples from the Array Collection (20 schizophrenia, 20 bipolar disorder, and 20 controls) consisting of the formalin-fixed DLPFC tissue was made available to Dr. Beasley's laboratory for performing microglia cell quantification.

In this study, we maximized the sample size for each analysis. That is, we included all samples that had both CMV antibody results and diagnostic information (analyses 1–2) and all samples that had both CMV antibody results and gene expression or microglia density data (analyses 3–4). See summary in Fig. 1.

CMV serostatus and IgG antibody levels

IgG antibodies against CMV were measured from serum using an enzyme-linked immunosorbent assay (ELISA, IBL America, catalog #EG101). A sample was generally considered CMV seropositive if it had an optical density (OD) value equivalent to approximately 20% over the supplied cutoff standard or a cutoff based on the distribution of the values.

To normalize data across batches, the OD values were quantified as plate-adjusted z-scores with a mean value of zero and a standard deviation of one. We further divided the IgG antibody levels into terciles based on IgG antibody percentile among the CMV seropositive samples (i.e., bottom: 0–33rd percentile, middle: 34–66th percentile, and upper: above 66th percentile).

Gene expression

The grey matter trimmed from the rostral-caudal 1/3 of the fresh frozen middle frontal gyrus was used. This tissue was pulverized, and the total RNA was extracted using TRIZOL. Total RNA was extracted from all six layers cortical layers of the middle frontal gyrus, at the level anterior to the genu of the corpus callosum, not including the frontal pole. White matter was removed from each block of tissue with a scalpel or razor blade before RNA extraction.

Gene expression changes for the cluster of these seven genes were confirmed with quantitative real-time polymerase chain reaction (qPCR) using an ABI Prism 7900HT Fast Real Time PCR system with a 384-well format for both targets and housekeeper normalizing controls (Applied Biosystems, Foster City, CA, USA) as previously reported [10, 29]. Specifically, pre-designed Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) were used to measure the following transcripts: SERPINA3 (Hs003153674_m1), IL6 (Hs00174131_m1), IL6ST (Hs01006741_m1), IL8 (HS00174103_m1), IL1B (Hs01555410_m1), NFKB1 (Hs00765730_m1), and PTGS2 (Hs00153133_m1). ACTB (Hs99999903_m1), GAPDH (Hs99999905_m1), TBP (Hs00427621_m1) and UBC (Hs00824723_m1) were used as normalizing transcripts and did not differ according to experimental groups.

Using a two-step recursive cluster analysis, we found that four of these seven transcripts (SERPINA3, IL-6, IL-8, and IL-1β) contributed significantly to the model and were thus used to define the 'High' vs 'Low' neuroinflammation groups [11]. Missing and outlier values were replaced using the SPSS-derived expectation maximization algorithm in order to retain as many cases as possible. The clustering was performed on all cases, and in order for the model quality to be considered acceptable, it had to exceed 0.5 on a scale of 0 to 1.0. Additionally, the individual predictor importance for each transcript had to exceed 0.4 on the same scale.

For the current study, we clustered a subset of the postmortem samples with both CMV antibody data and gene expression data ($n = 82$; 30 schizophrenia, 23 BD, and 29 controls) into "high" ($n = 30$, 13 schizophrenia, 8 BD, and 9 controls) or "low" inflammation groups ($n = 52$, 17 schizophrenia, 15 BD, and 20 controls) using the identical methodology described above.

Microglial cells

Microglia were quantified in a subset of 60 samples (20 schizophrenia, 20 BD, and 20 controls) by Dr. Beasley. For the current study, we excluded 3 participants with poor staining quality and 8 participants without CMV antibody data, resulting in data from 49 samples (17 controls, 19 with schizophrenia, and 13 with bipolar disorder). Tissue blocks were dissected from the DLPFC (BA9) gray matter, and paraffin embedded. Staining for ionized calcium-binding adaptor molecule-1 (IBA-1) was performed on three 6μm thick coronal sections, as previously described [30]. Briefly, sections were incubated overnight at 4 °C with a rabbit polyclonal antibody (Wako, Richmond, VA, USA; 1:8000), then with biotinylated goat anti-rabbit (Jackson Immunoresearch, West Grove, PA, USA; 1:1000), followed by peroxidase-conjugated streptavidin (Jackson Immunoresearch; 1:12000). IBA-1 was visualized using an enhanced ImmPACT DAB substrate

Table 1. Demographic and clinical details of postmortem samples.

CMV vs. diagnosis	Control	Schizophrenia	Bipolar Disorder	Depressive Disorder	*p*
n	85	114	78	87	
Age (Years, mean (SD))	45.54 (11.16)	44.24 (11.41)	45.23 (12.31)	41.78 (11.83)	0.142
Sex (Female (%))	21 (24.7)	34 (29.8)	37 (47.4)	31 (35.6)	0.014
PMI (h, mean (SD))	28.04 (13.15)	38.61 (24.22)	34.33 (14.61)	38.25 (25.14)	0.002
Brain pH (Mean (SD))	6.53 (0.29)	6.42 (0.28)	6.44 (0.30)	6.49 (0.27)	0.027
CMV serostatus Positive (%)	27 (31.8)	43 (37.7)	42 (53.8)	48 (55.2)	0.002
CMV antibody[a] (Mean (SD))	1.03 (0.76)	0.77 (0.56)	1.24 (0.62)	1.02 (0.55)	0.006
Ethnicity (Caucasian (%))	80 (95.1)	103 (90.4)	73 (93.6)	86 (98.9)	0.098
Manner of death (Suicide (%))	0 (0.0)	34 (29.8)	37 (48.1)	48 (55.8)	<0.001
CMV vs. suicide status	**Control**	**Schizophrenia**	**Bipolar Disorder**	**Depressive Disorder**	***p***
N	0	110	75	80	
Age (Years, mean (SD))	–	44.24 (11.34)	45.29 (12.28)	41.90 (12.00)	0.183
Sex (Female (%))	–	33 (30.0)	37 (49.3)	28 (35.0)	0.025
PMI[a] (h, mean (SD))	–	38.62 (24.62)	34.29 (14.77)	37.44 (25.29)	0.432
Brain pH (Mean (SD))	–	6.43 (0.28)	6.44 (0.30)	6.49 (0.28)	0.276
CMV serostatus Positive (%)	–	42 (38.2)	41 (54.7)	46 (57.5)	0.015
CMV antibody[b] (Mean (SD))	–	0.78 (0.56)	1.25 (0.63)	1.04 (0.55)	0.001
Ethnicity (Caucasian (%))	–	100 (90.9)	70 (93.3)	79 (98.8)	0.078
Manner of death (Suicide (%))	–	34 (30.9)	37 (49.3)	48 (60.0)	<0.001
CMV vs. neuroinflammation status	**Control**	**Schizophrenia**	**Bipolar Disorder**	**Depressive Disorder**	***p***
N	29	30	23	0	
Age (Years, mean (SD))	43.10 (7.83)	44.00 (7.61)	45.96 (9.68)	–	0.465
Sex (Female (%))	8 (27.6)	9 (30.0)	13 (56.5)	–	0.064
PMI[a] (h, mean (SD))	28.00 (13.33)	31.43 (16.76)	34.61 (15.20)	–	0.299
Brain pH (Mean (SD))	6.60 (0.28)	6.48 (0.24)	6.49 (0.28)	–	0.179
CMV serostatus Positive (%)	7 (24.1)	10 (33.3)	12 (52.2)	–	0.106
CMV antibody[b] (Mean (SD))	1.03 (0.92)	0.86 (0.65)	1.59 (0.75)	–	0.081
Ethnicity (Caucasian (%))	29 (100.0)	30 (100.0)	22 (95.6)	–	0.273
Manner of death (Suicide (%))	0 (0.0)	7 (23.3)	10 (43.5)	–	0.001
CMV vs. microglia activation	**Control**	**Schizophrenia**	**Bipolar Disorder**	**Depressive Disorder**	***p***
N	17	19	13	0	
Age (Years, mean (SD))	44.41 (6.56)	45.11 (6.92)	47.08 (9.53)	–	0.623
Sex (Female (%))	5 (29.4)	7 (36.8)	8 (61.5)	–	0.187
PMI[a] (h, mean (SD))	28.82 (13.15)	30.42 (11.43)	31.62 (11.91)	–	0.82
Brain pH (Mean (SD))	6.70 (0.25)	6.50 (0.23)	6.49 (0.32)	–	0.046
CMV serostatus Positive (%)	4 (23.5)	5 (26.3)	6 (46.2)	–	0.36
CMV antibody[b] (Mean (SD))	1.43 (1.02)	0.72 (0.49)	1.61 (0.80)	–	0.192
Ethnicity (Caucasian (%))	17 (100.0)	19 (100.0)	13 (100.0)	–	NA
Manner of death (Suicide (%))	0 (0.0)	4 (21.1)	4 (30.8)	–	0.06

[a]*PMI* Postmortem Interval.
[b]Only CMV seropositive samples.

(Vector Laboratories). All sections were counterstained with cresyl violet before being cover slipped. There were two counting frames per section (total of six), with counting frames extending from the pial surface to the grey-white matter border (1 mm in the x-axis and variable in the y-axis depending on cortical width). Delineation of cortical layers was based on cytoarchitectonic criteria of Rajkowska and Goldman-Rakic [31]. IBA-1 positive cells were categorized based on morphology and location into ramified, non-ramified or vascular subtypes by one experimenter, who was blind to diagnosis and CMV status. The intra-rater reliability of ramified and non-ramified cell counts, assessed by reanalyzing five cases chosen at random, was 0.84 and 0.97 respectively. Ramified cells displayed small, round soma with abundant thin, branched processes, while non-ramified cells displayed enlarged cell bodies with thickened processes that were fewer in number, or absent, and included primed, reactive and ameboid morphologies previously identified in human brain tissue [32]. Cells with soma located within or in contact with a blood vessel were classified as vascular and were not included in this study. The ratio of non-ramified to ramified microglia was used as a surrogate marker of microglial activation.

Statistical analysis

All statistical analysis were performed using R Statistical Software (version 4.1.3; R Core Team 2021). The Shapiro-Wilk's test was performed to confirm the normality of continuous variables. We first calculated the prevalence of CMV seropositivity among control and psychiatric disorder groups. Second, a multivariable logistic regression model was used to test whether CMV

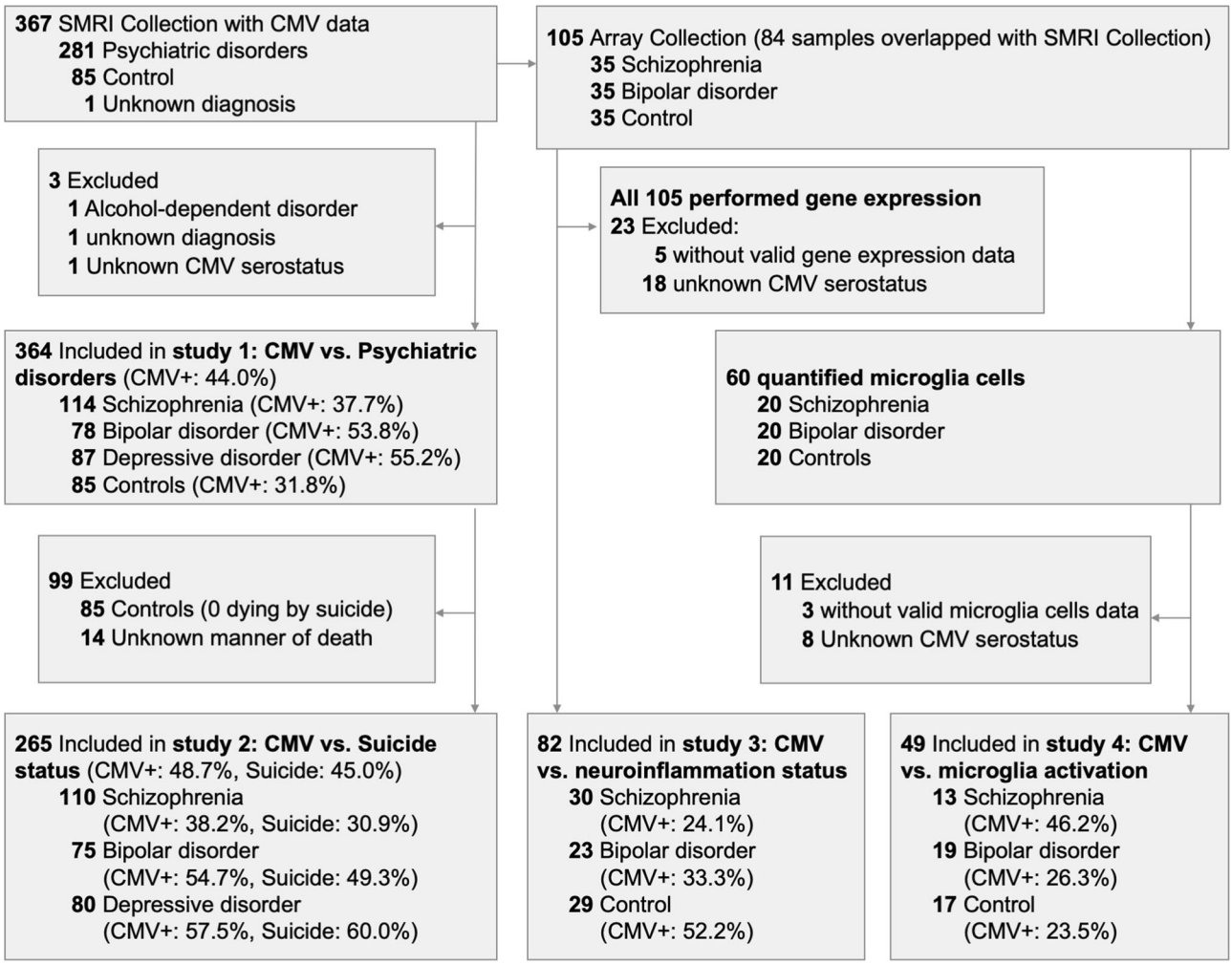

Fig. 1 Sample inclusion and exclusion flowchart. The figure shows the detailed information for the final sample included in each analysis.

serostatus and CMV antibody IgG levels were associated with the likelihood of having a psychiatric disorder or dying by suicide. The odds ratios (ORs) and 95% confidence intervals (95%CIs) were estimated from the multivariable logistic regression model. The same multivariable logistic regression models were performed as post hoc tests to assess which specific psychiatric disorder accounted for the observed results. The possible confounders, age, sex, and ethnicity were included as covariates in the models. Third, a multivariable logistic regression model was used to test whether CMV seropositivity and CMV antibody IgG levels increased the odds of being assigned to the "high" vs. "low" inflammation groups. In addition to age, and sex, PMI, brain acidity (pH), and diagnosis was also controlled for in this model as psychiatric disorders were previously linked to differences in inflammation-related gene expression [10]. Fourth, multivariable linear regression models were used to investigate the association between CMV serostatus and microglia activation (ratio of non-ramified to ramified microglia) controlling for age, sex, PMI, pH, and diagnosis. We did not control for ethnicity for neuroinflammation and microglia-related analyses because the samples were 99% Caucasian (also see Table 1). Sub-group analyses using the same models were performed for the association between CMV serostatus, neuroinflammation status, and microglia activation to assess which specific diagnostic group accounted for the observed results. Finally, we conducted sensitivity analyses using the aforementioned models but without controlling for brain pH. This is because decreased brain pH has been linked with psychiatric disorders [33] and neuroinflammation [34]. Therefore, in addition to our primary statistical model (which may be overly conservative by reducing some of the inflammatory effects), we conducted sensitivity analyses without controlling for brain pH. We also performed sensitivity analyses controlling for medical comorbidity. The variance inflation factor was computed to assess model multicollinearity in all linear regression models.

RESULTS

CMV seropositivity rates across the diagnostic groups

As summarized in Table 1, the prevalence of CMV seropositivity was higher in samples with a psychiatric disorder (133/279, 47.7%) than controls (27/85, 31.8%). Among the samples with a psychiatric disorder, samples with a depressive disorder showed the highest CMV prevalence (48/87, 55.2%), followed by BD (44/78, 53.8%), and schizophrenia (43/114, 37.7%).

CMV serostatus, IgG antibody levels and psychiatric disorder

Relative to CMV seronegative samples, CMV seropositive samples were more than twice as likely to have a psychiatric disorder (OR = 2.19, 95%CI = 1.27–3.87, $p = 0.006$) after controlling for confounders (Fig. 2A). Post-hoc analysis (Supplementary Table S3) revealed that CMV seropositivity did not significantly increase the odds of having schizophrenia (OR = 1.40, 95%CI = 0.75–2.65, $p = 0.30$). However, CMV seropositivity significantly increased the odds of having a mood disorder (bipolar disorder: OR = 2.45, 95% CI = 1.24 – 4.93, $p = 0.01$; depressive disorder: OR = 3.70, 95%CI: 1.84 – 7.74, $p < 0.001$).

To test for an association between CMV IgG antibody levels and the risk of having a psychiatric disorder, we grouped antibody levels into bottom, middle, and upper terciles. Relative to CMV seronegative samples, samples in the bottom tercile had 72% increased odds of having a psychiatric disorder which was not statistically significant (OR = 1.72, 95%CI = 0.80–3.98, $p = 0.184$).

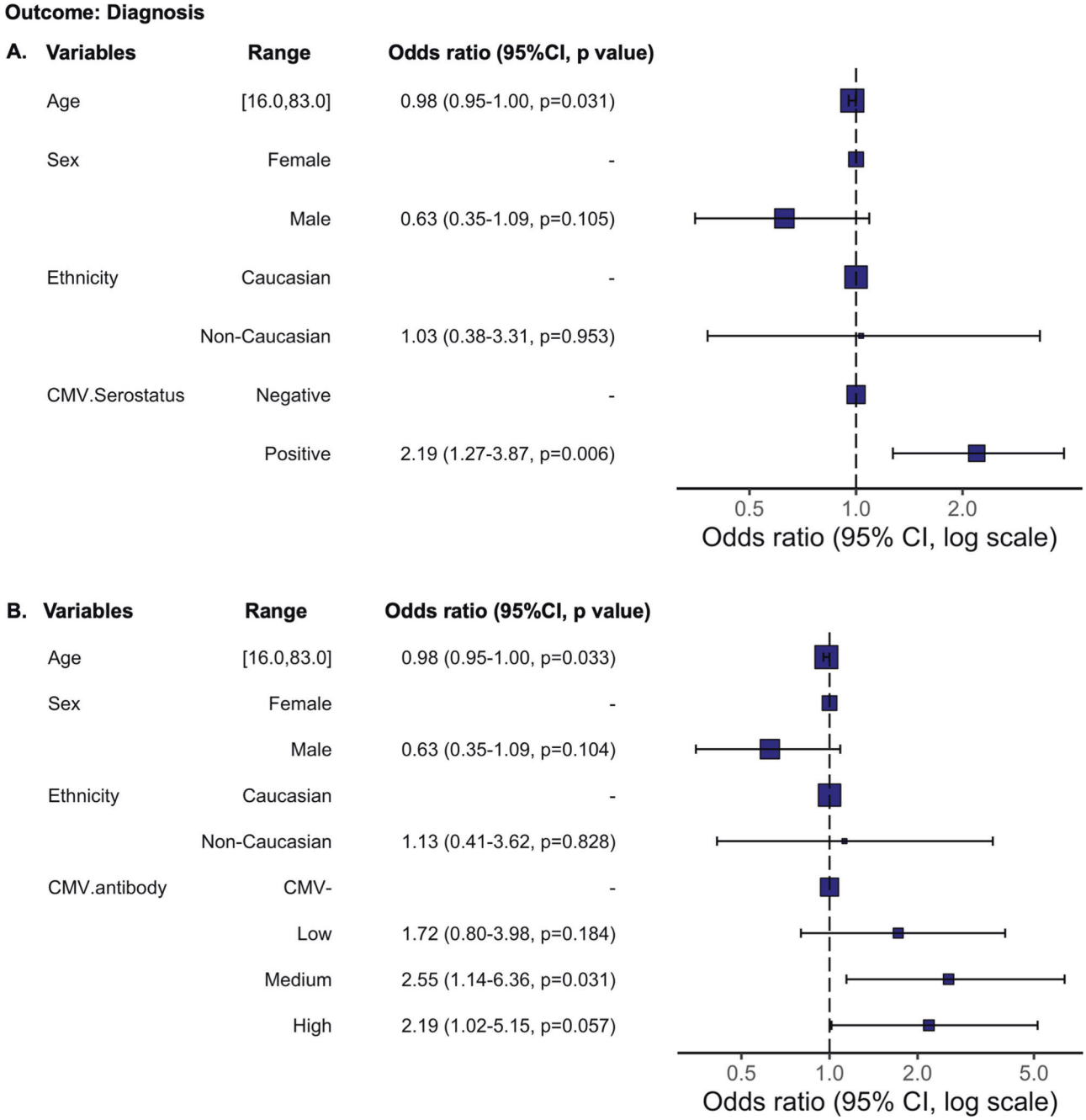

Fig. 2 Associations between CMV infection and psychiatric disorders. A Relative to CMV seronegative samples, CMV seropositive samples are associated with increased odds of having a psychiatric disorder (defined as a binary outcome: having a psychiatric disorder diagnosis was coded as 1, and control samples were coded as 0). **B** CMV seropositive samples were grouped into 3 groups (low, medium, and high) based on the percentile of antibody IgG levels. Relative to CMV negative samples, medium and high CMV antibody IgG levels are associated with significantly increased odds of having a psychiatric disorder.

Samples in the middle and upper terciles were more than twice as likely to have a psychiatric disorder (middle: OR = 2.55, 95% CI = 1.14–6.36, $p = 0.031$; upper: OR = 2.19, 95%CI = 1.02–5.15, $p = 0.057$) after controlling for confounders (Fig. 2B). Post-hoc subgroup analyses revealed that CMV antibody levels did not significantly increase the odds of having schizophrenia (OR ranged from 0.97 to 1.59). However, CMV antibody levels in the medium and high terciles significantly increased the odds of having a mood disorder (bipolar disorder: antibody level medium OR = 3.64, 95%CI = 1.39 – 10.28, $p = 0.01$, antibody level high OR = 2.89, 95%CI = 1.15 – 7.68, $p = 0.02$; depressive disorder: antibody level medium OR = 4.36, 95%CI = 1.62 – 12.77, $p = 0.005$, antibody level high OR = 3.90, 95%CI = 1.54 – 10.54, $p = 0.005$, Supplementary Table S3). The variance inflation factor for each covariate ranged from 1.00 to 1.15 suggesting that multicollinearity was not a concern in the models.

CMV serostatus, IgG antibody levels, and suicide

Among those individuals with a psychiatric disorder, CMV seropositive samples were more than twice as likely to die by suicide (OR = 2.09, 95%CI = 1.27–3.48, $p = 0.004$) relative to CMV seronegative samples, after controlling for potential confounders

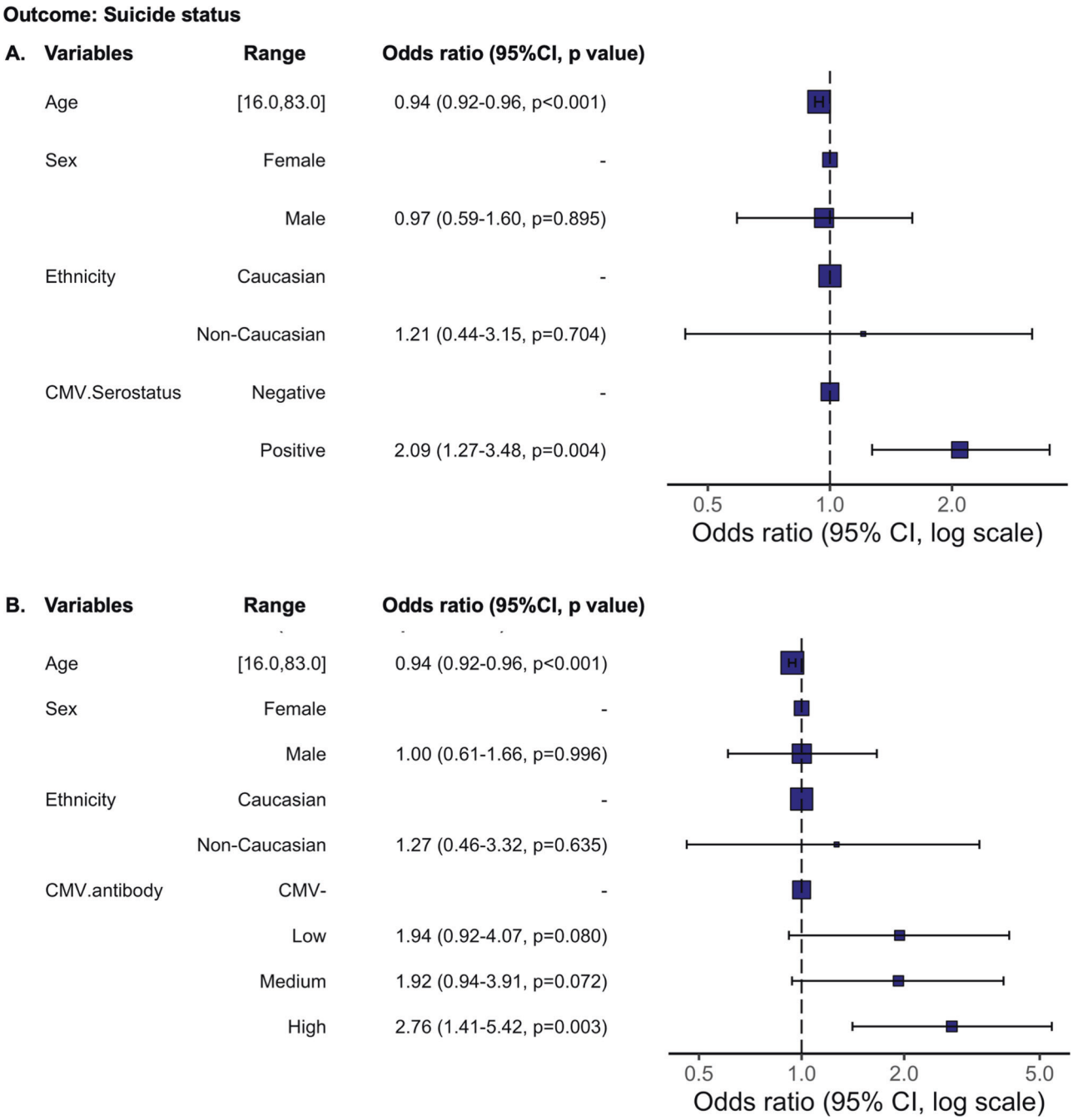

Fig. 3 Associations between CMV infection and suicide status. A Relative to CMV seronegative samples, CMV seropositive samples are associated with an increased likelihood of dying by suicide. **B** CMV seropositive samples were grouped into 3 groups (low, medium, and high) based on the percentile of antibody IgG levels. Relative to CMV negative samples, both low and high CMV antibody IgG levels are associated with an increased likelihood of dying by suicide.

(Fig. 3A). Post hoc sub-group analyses showed that the effect size was similar across all three psychiatric disorders (namely, schizophrenia, bipolar disorder, and depressive disorder) with OR ranging from 1.20 to 1.70 but not statistically significant in any of the groups (Supplementary Table S4).

We also tested for an association between CMV IgG antibody levels and suicide within the psychiatric disorder group. Relative to CMV seronegative samples, the odds of dying by suicide increased more than 90% for seropositive samples in the bottom tercile and middle tercile groups (bottom tercile group: OR = 1.94, 95%CI = 0.92–4.07, $p = 0.080$; middle tercile group: OR = 1.92, 95%CI = 0.94–3.91, $p = 0.072$) but was not statistically significant. Samples in the upper tercile were almost three times more likely to die by suicide (OR = 2.76, 95%CI = 1.41–5.42, $p = 0.003$) as compared with CMV seronegative samples after controlling for potential confounders (Fig. 3B). Additional sub-group analyses showed that the magnitude of the effect of CMV antibody levels was comparable for all three psychiatric disorders. Specifically, the odds ratios (OR) for the effect size ranged from 0.44 to 4.02, but these results were not statistically significant in any of the subgroups, as shown in Supplementary Table S4. The variance inflation factor for each covariate ranged from 1.01 to 1.18 suggesting that multicollinearity was not a concern in the models.

Table 2. Comparison of primary analyses and sensitivity analyses results.

	Models controlling for brain pH (Primary analyses)			Models not controlling for brain pH (Sensitivity analyses)		
CMV vs. neuroinflammation status ($n_{total} = 82$, $n_{CMV+} = 29$, $n_{high\ neuroinflammation} = 30$)	OR	95% CI	*p*	OR	95% CI	*p*
CMV seropositivity	1.81	0.54–6.20	0.333	2.10	0.74–6.12	0.163
CMV antibody levels low	0.35	0.02–2.69	0.379	0.39	0.02–2.61	0.405
CMV antibody levels medium	2.24	0.26–19.21	0.452	2.94	0.55–16.72	0.203
CMV antibody levels high	4.41	0.93–23.64	0.068	4.20	1.09–18.24	**0.042***
CMV vs. microglia activation ($n_{total} = 49$, $n_{CMV+} = 15$)	Cohen's d	95% CI	*p*	Cohen's d	95% CI	*p*
dlPFC Layer I	0.81	0.14–1.48	**0.024***	0.83	0.16–1.49	**0.019***
dlPFC Layer II	0.56	−0.16–1.27	0.136	0.56	−0.14–1.26	0.126
dlPFC Layer III	0.40	−0.34–1.13	0.299	0.40	−0.32–1.13	0.281
dlPFC Layer IV	0.51	−0.22–1.23	0.176	0.48	−0.23–1.19	0.194
dlPFC Layer V	0.58	−0.15–1.31	0.125	0.56	−0.16–1.27	0.133
dlPFC Layer VI	0.31	−0.42–1.04	0.412	0.29	−0.44–1.01	0.443
dlPFC All Layers	0.56	−0.17–51.28	0.141	0.55	−0.16–1.30	0.137

The ratio of non-ramified to ramified microglia was used as a surrogate marker of microglial activation. OR, odds ratio; 95% CI, 95% confidence interval.

CMV serostatus, IgG antibody levels, and neuroinflammation

There was no significant relationship between CMV serostatus and inflammation-related gene expression levels (Table 2, and Supplementary Table S5). However, relative to CMV seronegative samples, samples with anti-CMV antibody levels in the upper tercile were more than four times more likely to have high inflammation-related gene expression (OR = 4.41, 95%CI = 0.93 – 23.64, $p = 0.068$, Table 2). Due to the smaller sample size and wide confidence intervals, this association only trended significant (although it was statistically significant when brain pH was omitted from the model – see Table 2). The observed effect was likely driven by schizophrenia and bipolar disorder subgroups as shown in Supplementary Table S5. The variance inflation factor for each covariate ranged from 1.09 to 1.21 suggesting that multicollinearity was not a concern in the models.

CMV serostatus and microglia activation

Relative to CMV seronegative samples, CMV seropositive samples showed an increase in the ratio of non-ramified to ramified microglia (Fig. 4A and 4B) in all six layers of the DLPFC combined and each of the six layers individually although the effect only reached statistical significance in layer I (Cohen's d = 0.81, standard error=0.34, $p = 0.023$, Table 2, and Fig. 4C). When we performed subgroup analyses, we found that CMV seropositivity was associated with a moderate effect of increased microglia activation in layer I of DLPFC. However, none of the groups reached statistical significance, as shown in Supplementary Table S6. To avoid generating heavily biased results using extremely small samples (i.e., 3 samples in bottom tercile group, 6 samples in the middle tercile group, and 6 samples in the upper tercile group), we did not run the analyses to test for associations between CMV antibody levels and microglia activation. The variance inflation factor for each covariate ranged from 1.16 to 1.37 suggesting that multicollinearity was not a concern in the models.

Sensitivity analyses

Sensitivity analyses using similar statistical models but without controlling for brain pH showed consistent results with similar effect sizes (Table 2) supporting the robustness of the findings reported above. Similarly, when we accounted for medical comorbidity, all the conclusions remained the same. The results are shown in Supplementary Table S7.

DISCUSSION

To our knowledge, this is the first study to examine the correlates of CMV infection at the time of death within the context of psychiatric illness. There were three main results. First, samples from individuals with CMV seropositivity were significantly more likely to have BD (OR = 2.45) or a unipolar depressive disorder (OR = 3.70) than individuals who were CMV seronegative. The effect appeared to be driven by samples with antibody levels in the middle and upper terciles. Second, among the samples with a psychiatric disorder, those individuals who tested CMV seropositive were significantly more likely to die by suicide than the individuals who tested seronegative for CMV (OR = 2.09). The effect appeared to be driven by samples in the upper antibody tercile who were approximately three times more likely to be suicides than CMV negative samples. Third, the CMV seropositive group displayed a greater ratio of non-ramified to ramified microglia in layer I of the DLPFC, and consistent with this putative indicator of inflammation, individuals in the upper antibody tercile were more than four times more likely than CMV negative samples to be members of the "high" inflammation group, which was previously shown to have increased numbers of reactive astrocytes and elevated cytokine concentrations compared with the "low" inflammation group [10, 11].

The association between CMV seropositivity and mood disorders is broadly consistent with the epidemiological literature. At least 14 observational studies have linked CMV infection with unipolar depression [17, 18, 35–46]. Less work has been done on BD, but one case-control study reported higher CMV seropositivity rates in 1,200 participants with BD relative to 745 healthy controls [47]. Also notable are two large prospective studies which found that CMV seropositivity was associated with an increased risk of future depression [17] or mood disorders, more generally [18]. Other studies have reported a link between CMV antibody titers and mood disorders. Anti-CMV titers are a surrogate marker of viral reactivation, with higher antibody levels generally indicative of an active infection [48]. For instance, the Detroit Neighborhood Health Study showed that for every one unit increase in CMV IgG antibody titer, the odds of incident depression increased by 26% so that individuals with IgG antibody titers in the highest quartile had four times greater odds of depression compared with participants in the lower three quartiles [41]. In the case of schizophrenia, results have been more equivocal [14] which may explain the negative findings of this study. Although some early

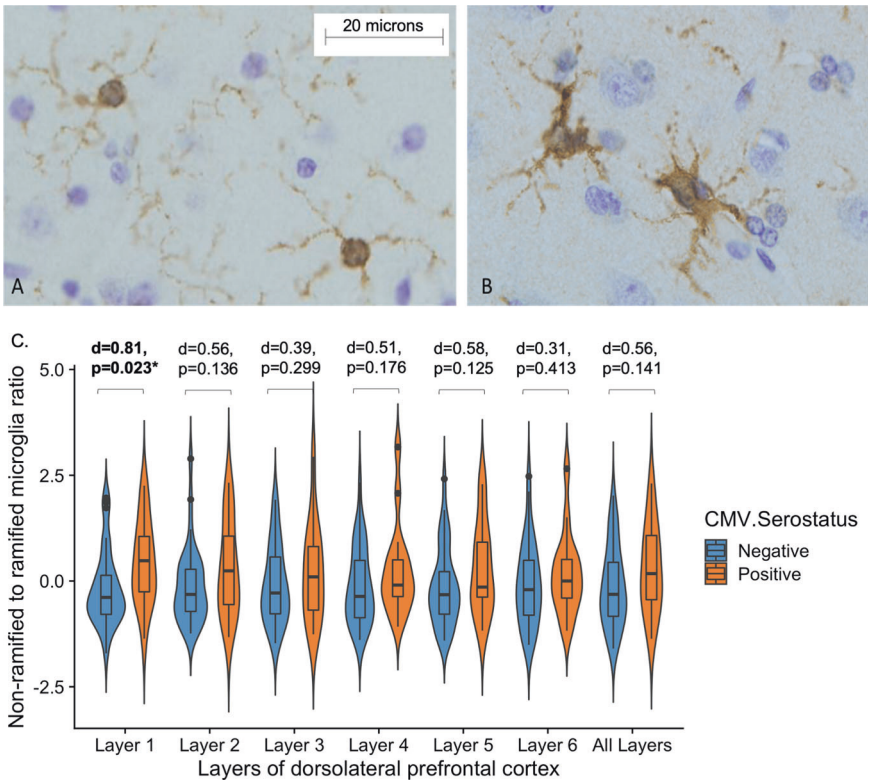

Fig. 4 Associations between CMV infection and microglia activation. A Ramified microglia displayed small, round cell bodies with numerous thin, branched processes, whereas (**B**), non-ramified microglia displayed enlarged or amorphous soma, and processes that were thickened, fewer in number or absent. **C** CMV seropositivity is associated with an increased ratio of non-ramified to ramified microglia. Note that the figure illustrates the ratio after regressing out the effects of age, PMI, sex, brain pH, and diagnosis.

papers reported a higher frequency of CMV seropositivity in schizophrenia [49–51], these findings proved challenging to replicate and several meta-analyses or systematic reviews have failed to detect a statistical association between CMV serostatus and schizophrenia [52–54]. However, since heightened neuroinflammation is observed in a subset of people with schizophrenia, a more stratified approach may be required to test the association between schizophrenia and CMV IgG antibody titers in the future.

Several published studies support the link between CMV infection and suicide. A case-control study including over 80,000 Danish blood donors found that CMV seropositivity was associated with an increased risk of attempting or dying by suicide [18]. Similarly, Dickerson and colleagues followed over 1,000 individuals with schizophrenia, BD, and major depressive disorder over 8 years and found that increasing levels of CMV antibodies were associated with increasing hazard ratios for suicide [42]. Another group reported higher IgG anti-CMV antibody levels in depressed patients with at least two suicide attempts compared with depressed participants with no history of suicide attempts [45]. Nevertheless, it should be noted that not all studies support the link between CMV and suicide [55, 56] while another paper detected an association between suicide and anti-CMV IgM titers but not IgG titers [40]. The results of the current paper provide support to those epidemiological studies that have reported a positive association between CMV and suicide.

At "rest", microglia display a ramified morphology whereas upon activation they display a graded series of morphological changes leading up to a non-ramified, ameboid-like phenotype [57, 58]. Here, we used the ratio of non-ramified to ramified microglia as a surrogate marker of microglia activation. The increased ratio of non-ramified to ramified microglia in the CMV positive versus the CMV negative samples in layer I of DLPFC is consistent with preclinical studies demonstrating that microglia play an important role in protecting the brain against CMV infection [59]. Similarly, murine models of congenital CMV infection are characterized by elevated levels of microglia-derived chemokines, infiltration of leukocytes, and activation of microglia in the brain [60–62]. In addition, in humans, CMV has been shown to cause microglial nodular encephalitis in the context of HIV infection [63, 64]. Although the difference in the ratio of non-ramified to ramified microglia density between CMV positive and CMV negative samples was only statistically significant in the most superficial cortical layer, we found this ratio to be greater in the CMV positive group in all six cortical layers (Fig. 4). For most of the layers the effect size ranged from 0.4 to 0.6 (Cohen's D), suggesting a medium effect size. Thus, we suggest that the microglial activation is likely to be anatomically generalized but larger sample sizes are required to confirm this hypothesis.

The microglia result together with the finding that samples in the upper tercile of CMV antibody levels were more than four times more likely than CMV negative samples to be assigned to the "high" neuroinflammation group (which in previously published work showed higher cytokines, increased astrogliosis, and greater HLA-DR$^+$ cell density) is suggestive of at least two possibilities. First, that reactivation of CMV is a cause of neuroinflammation or second, that neuroinflammation triggers CMV reactivation. The first possibility is consistent with work in other fields showing that CMV infection may trigger graft rejection and reduce the survival of transplant recipients [65] as well as contribute to medical morbidity in the context of HIV [13], sepsis [66], and COVID-19 [67, 68]. Further, treatment of HIV positive patients with the anti-CMV medication, valganciclovir, was demonstrated to reduce CD8+ (CD38$^+$ HLA-DR$^+$) cell immune activation as well as plasma concentrations of sTNFR2, sCD163, and sCD14 compared with placebo [13, 69]. On the other hand, it

is well established that inflammatory mediators such as tumor necrosis factor (TNF) and interleukin 6 (IL-6) promote CMV reactivation via AP-1 or NF-kB-induced transcription of the immediate early CMV promoter [6, 70]. Experimental designs are required to disambiguate the relative contributions of these opposing processes. However, our hypothesis is that both phenomena are likely at play. That is, inflammation reactivates CMV, and lytic viral replication in turn exacerbates the underlying inflammation and conceivably helps translate peripheral inflammation into neuroinflammation.

This study has several limitations. First, serum was not available for all samples in the SMRI brain bank and thus CMV serostatus could not be determined for all samples. As a result, the gene expression and histological analyses may have been underpowered, especially as we elected to control for up to six potential confounders in the statistical models, an approach that is not always taken in the postmortem literature because of sample size limitations. Second, the samples included in the current study were predominantly Caucasian (93.2%). Therefore, the results may not generalize to other populations. Third, there are several other neurotrophic viruses that may play a role in psychiatric illness. While our primary focus has been on CMV because of its association with neurological disease [1] and its significant impact on the immune system [71], future studies should examine the potential effects of other viral agents. Fourth, samples were labeled as showing “high” or “low” inflammation based on the expression of four different genes (two cytokines, a chemokine, and a serine protease inhibitor found in reactive astrocytes [72]) that provided a snapshot of immune system activity rather than capturing the full extent of inflammatory signaling. Fifth, there was a greater incidence of medical comorbidity in the CMV positive samples. Although sensitivity analyses showed that the key results remained unaltered after statistical control for medical disorders, there was significant missing information (~45%). This may have reduced the rigor of these sensitivity analyses. Lastly, the distinction between non-ramified and ramified microglia density should be viewed as heuristic since microglia are thought to display a continuum of activation rather than a simple binary “on” or “off” phenotype.

In sum, the current study raises the possibility that CMV is a risk factor for mood disorders and suicide through its possible neuroinflammatory effects. Further research is necessary to follow-up on these initial results to determine whether CMV is playing a causal role in psychiatric illness. If CMV does indeed contribute to neuroinflammation in the context of mental illness, then this may open-up a novel avenue of treatment given the existence of approved medications for the treatment of CMV [73–75].

DATA AVAILABILITY

Data used in current study are available at https://stanleyresearch.org. The statistical analysis R scripts used for current study are available upon request to the corresponding author.

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AUTHOR CONTRIBUTIONS

Conceptualization, HZ, MJW, MPP, RHY, and JS; data collection, MJW, CSW, CLB, and RHY; methodology and data analysis, HZ, MJW, CSW, CLB, and RHY; manuscript

writing—original draft preparation, HZ, and JS; manuscript writing—review and editing, HZ, MJW, CSW, CLB, MPP, RHY, and JS; All authors have read and agreed to the published version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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