




## RESEARCH ARTICLE

# Association between infectious burden and cerebral microbleeds: a pilot cross-sectional study

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## Abstract

**Objective:** Cerebral microbleeds (CMBs) is a subtype of cerebral small vessel disease. Their underlying pathogenesis remains unclear. The aim of this study was to investigate the association between infectious burden (IB) and CMBs.

**Methods:** Seven hundred and seventy-three consecutive patients who were hospitalized in the Department of Neurology in General Hospital of Western Theater Command without severe neurological symptoms were recruited and selected in this pilot cross-sectional study. CMBs were assessed using the susceptibility-weighted imaging sequence of magnetic resonance imaging. Immunoglobulin G antibodies against common pathogens, including herpes simplex virus (HSV)-1, HSV-2, cytomegalovirus (CMV), *Chlamydia pneumoniae* (*C. pneumoniae*), *Mycoplasma pneumoniae* (*M. pneumoniae*), Epstein-Barr virus (EBV), *Helicobacter pylori* (HP), and *Borrelia burgdorferi* (*B. burgdorferi*), were measured by commercial ELISA assays. IB was defined as a composite serologic measure of exposure to these common pathogens. **Results:** Patients with and without CMBs were defined as the CMBs group ( $n = 76$ ) and the non-CMBs group ( $n = 81$ ), respectively. IB was significantly different between the CMBs and non-CMBs groups. After adjusted for other risk factors, the increased IB was independently associated with the presence of CMBs ( $P = 0.031$ , OR = 3.00, 95% CI [1.11–8.15]). IB was significantly positively associated with the number of CMBs (Spearman  $\rho = 0.653$ ,  $P < 0.001$ ). The levels of serum inflammatory markers were significantly different between the CMBs and non-CMBs groups and among the categories of IB. **Interpretation:** IB consisting of HSV-1, HSV-2, CMV, *C. pneumoniae*, *M. pneumoniae*, EBV, HP, and *B. burgdorferi* was associated with CMBs. All the findings suggested that pathogen infection could be involved in the pathogenesis of CMBs.

## Introduction

Cerebral microbleeds (CMBs) are small hypointense lesions that are visible on paramagnetic-sensitive sequences of magnetic resonance imaging (MRI), and are located most commonly in the cortico-subcortical junction, deep grey, and white matter in the cerebral hemispheres, cerebellum, and brainstem.<sup>1</sup> Generally, CMBs are frequently detected in patients with hypertensive arteriopathy and cerebral amyloid angiopathy.<sup>2</sup> An increased number of CMBs can indicate a high risk for cerebral hemorrhage, cerebral ischemia, and cognitive decline.<sup>3,4</sup> Moreover, CMBs are also observed in the healthy elderly people with a prevalence of 11.1–23.5%, which increases with age.<sup>2</sup> However, the underlying pathogenesis of CMBs is poorly understood.

Single pathogen infection has been shown to be closely associated with vascular disease in previous studies. One study showed that CagA-positive *Helicobacter pylori* (HP) infection promoted macrophage-derived foam cell formation and augmented atherosclerotic plaque growth and instability.<sup>5</sup> Seropositivity toward herpes simplex virus (HSV) – 2 has been reported to be associated with myocardial infarction and stroke before the age of 50.<sup>6</sup> Moreover, it has been shown that the presence of cytomegalovirus (CMV) DNA increases the risk of both ischemic and hemorrhagic stroke in the Chinese population.<sup>7</sup> Additionally, elevated *Chlamydia pneumoniae* (*C. pneumoniae*) IgA titers are associated with an increased risk of ischemic stroke.<sup>8</sup>

Currently, infectious burden (IB) is an emerging concept, which is defined as the composite serological measure of exposure to multiple common pathogens.<sup>9</sup> Similar to the single pathogen infection, several studies have revealed that IB is also associated with vascular disease. One study showed that IB is significantly associated with the extent of atherosclerosis and increased the risk of future death in patients with advanced atherosclerosis.<sup>10</sup> Others have shown that IB is associated with the risk of first-ever stroke and the long-term prognosis of coronary artery disease, respectively.<sup>9,11</sup>

CMBs is a type of vascular disease, but the association between CMBs and IB remains unknown. Therefore, in this study, we compared IB between patients with CMBs and those without CMBs, explored the association between single pathogen infection or IB and the presence of CMBs, investigated the association between IB and the number and location of CMBs, and compared the levels of serum inflammatory markers between patients with CMBs and those without CMBs and among categories of IB. Our findings suggest that pathogen infection could be involved in the pathogenesis of CMBs.

## Methods

### Study design and patients selection

This study was approved by the Ethical Review Board of the General Hospital of Western Theater Command (No. 2018ky06) and registered in the Chinese Clinical Trial Registry (No. ChiCTR1800020330). Consecutive patients who were hospitalized in the Department of Neurology in this hospital were recruited from November 2017 to April 2019. The inclusion criteria of the patients were as follows: (1) age  $\geq$  45 years; (2) without severe neurological symptoms; and (3) patients or their direct family members were willing to sign informed consent. The patients were excluded if they<sup>12</sup>: (1) did not undergo all the four sequences of MRI including T1-weighted, T2-weighted, fluid attenuated inversion recovery (FLAIR), and susceptibility weighted imaging (SWI); (2) suffered from the clinical diseases and conditions which possibly influence the detection of antibodies against pathogens or inflammatory markers in the blood; and (3) suffered from severe neurologic diseases which possibly influence the assessment of MRI images.

### Assessment of clinical and laboratorial characteristics

The clinical and laboratory characteristics of the two groups were recorded, which included gender; age; body mass index (BMI); smoking history; alcohol intake history; hypertension; systolic blood pressure (SBP); diastolic blood pressure (DBP); diabetes mellitus; hyperlipidemia; ischemic heart disease (IHD); antiplatelet/anticoagulant use; and the blood levels of fasting glucose, glycated hemoglobin (HbA1c), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, and total cholesterol. Blood pressure was measured in two readings using a random sphygmomanometer 5 min after the patients rested, and both measures were averaged. Hypertension was defined as SBP  $\geq$  140 mmHg, and/or DBP  $\geq$  90 mmHg, self-reported hypertension history, or use of antihypertensive medication. Diabetes mellitus was defined as serum fasting glucose level  $\geq$  7.0 mmol/L, HbA1c level  $\geq$  6.5%, self-reported diabetes mellitus history, or use of antidiabetic medication. Hyperlipidemia was defined as LDL level  $\geq$  3.62 mmol/L, total cholesterol level  $\geq$  5.69 mmol/L, triglycerides level  $\geq$  1.69 mmol/L, self-reported hyperlipidemia history, or use of lipid-lowering therapy. Blood levels of fasting glucose, HbA1c, LDL, HDL, triglycerides, and total cholesterol were tested in the Department of Clinical Laboratory.

## Brain MRI scanning

We performed a multisequence MRI protocol on a 3.0T scanner (Philips, Netherlands). The SWI sequence was used for CMBs detection using the following parameters: fractional anisotropy, 10; repetition time, 13–15 msec; echo time, 19–21 msec; slice thickness, 1.2 mm; slice gap, 0 mm; field of view, 220 mm. The other sequences in the protocol comprised three high-resolution axial scans including T1-weighted, T2-weighted, and FLAIR sequences.

## Assessment of brain MRI images

All MRI images were assessed by two well-trained neuro-radiologists who were blinded to this study. On the SWI sequence, CMBs were visualized as small, homogeneous, low-signal, round or ellipse shapes.<sup>1</sup> In general, the diameter of a single microbleed lesion was in the range 2–10 mm and the focal area was not detected on CT or routine MRI sequences.<sup>1</sup> Microbleed mimics such as vessel cross-section, flow voids, microcalcifications, iron deposition from other causes, and microaneurysms were excluded. The number and location of the CMBs were recorded. All CMBs were categorized into two distinct anatomical regions: lobar (cortical, subcortical) and deep/infratentorial (D/I) (basal ganglia, thalamus, internal capsule, external capsule, extreme capsule, deep or periventricular white matter, cerebellum, and brain stem).<sup>13</sup> In addition, lacunes and white matter hyperintensity (WMH) were assessed using FLAIR, T1-weighted, and T2-weighted sequences. Lacunes were defined as round or ovoid, subcortical, hypointense lesions with a hyperintense rim on FLAIR images, which was 3–15 mm in diameter. WMH was hyperintense on T2-weighted/FLAIR sequences and could appear as isointense or hypointense on T1-weighted sequences.<sup>1</sup> The degree of WMH was visually evaluated on FLAIR images using the Fazekas scale with periventricular WMH and deep WMH being rated separately. The total Fazekas score was the sum of periventricular and deep WMH, ranging from 0 to 6.<sup>14</sup> Inter-rater reliability for the presence of CMBs was Kappa = 0.86, and for the number of CMBs, the intra-class correlation coefficient was 0.96.

## Assessment of IB and serum inflammatory markers

Following previous studies,<sup>10,15</sup> IB was measured by aggregating the number of single seropositivities for HSV-1, HSV-2, CMV, *C. pneumoniae*, *Mycoplasma pneumoniae* (*M. pneumoniae*), Epstein–Barr virus (EBV), HP, and *Borrelia burgdorferi* (*B. burgdorferi*), respectively. Blood

was sampled from the antecubital vein after admission and centrifuged at 1200 g at 4°C for 10 min immediately. Subsequently, the serum was stored at –80°C. Pathogen serologies were measured using immunoglobulin G enzyme-linked immunosorbent assay (ELISA) kits for HSV-1 (JL45461, J&L, China), HSV-2 (JL45463, J&L, China), CMV (JL18486, J&L, China), *C. pneumoniae* (JL46233, J&L, China), *M. pneumoniae* (JL46234, J&L, China), EBV (JL46275, J&L, China), HP (JL14030, J&L, China), and *B. burgdorferi* (JL46263, J&L, China). Serum inflammatory markers, including C-reactive protein (CRP) (JL46268, J&L, China), interferon (IFN) - $\gamma$  (JL12152, J&L, China), interleukin (IL)-1 $\beta$  (JL29721, J&L, China), IL-6 (JL10457, J&L, China), and tumor necrosis factor (TNF)- $\alpha$  (JL10208, J&L, China), were measured using commercial ELISA kits. All the above ELISA measurements were performed in a blinded manner to clinical outcomes.

## Statistical analysis

Statistical analysis was performed using SPSS version 23.0. Categorical variables were expressed as counts (%) and continuous variables were expressed as mean (standard deviation [SD]) or median (interquartile range [IQR]) values. Baseline characteristics and serum inflammatory markers between the CMBs and non-CMBs groups were compared by independent sample *t* test, Mann–Whitney *U* test, or chi-square test as appropriate. The chi-square test was applied to compare the proportion of IB between the two groups and to analyze the association between IB and the location of CMBs. Logistic regression analysis was used to analyze the association between a single pathogen infection or IB and the presence of CMBs. In addition to gender and age, all clinical variables with *P* < 0.20 in the univariate analysis were incorporated into the logistic regression analysis. The association between IB and the number of CMBs was determined by Spearman rank correlation analysis. The Kruskal–Wallis one-way ANOVA analysis was used to compare the levels of serum inflammatory markers between the two groups and among categories of IB. Confidence intervals (CIs) at the 95% level were calculated for the odds ratio (OR). *P* values < 0.05 were considered statistically significant.

## Results

### Baseline characteristics of patients

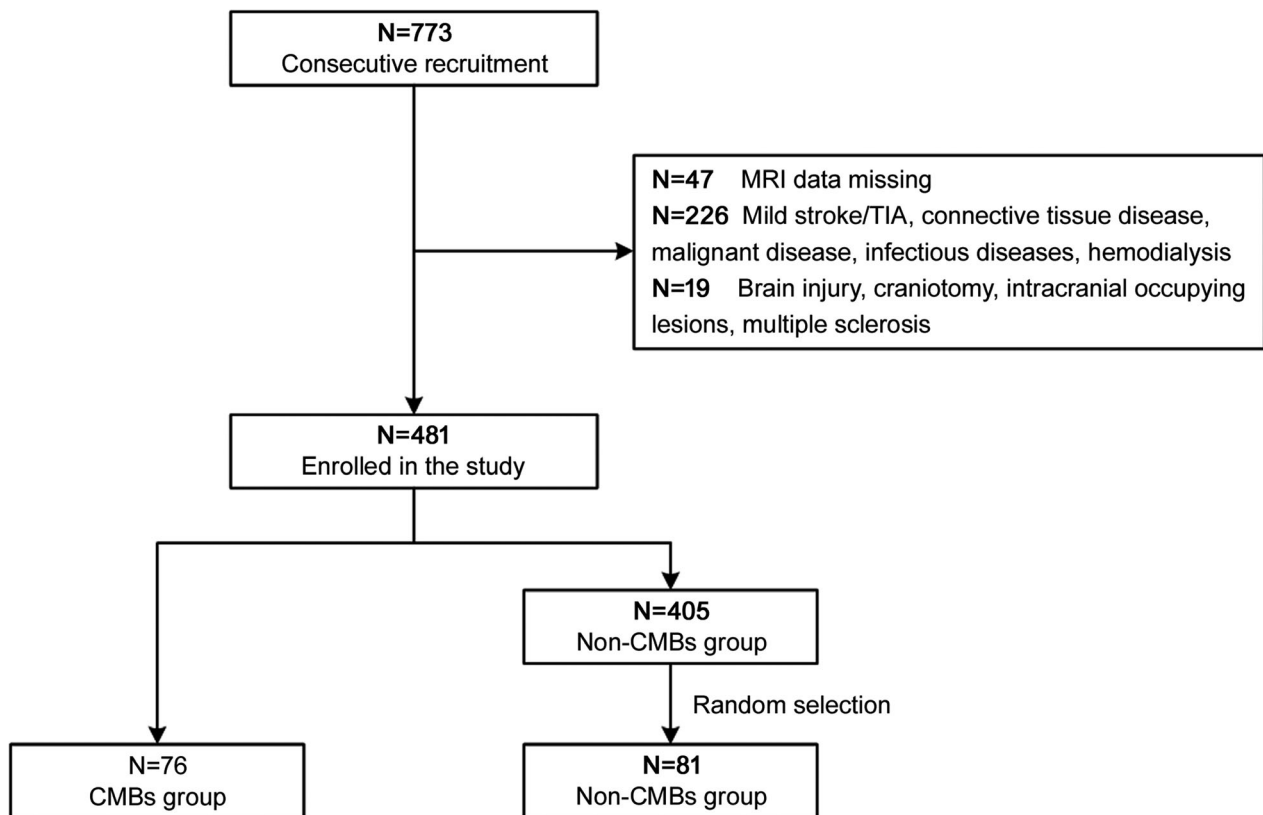
In total, 773 patients were recruited, and the main clinical features manifested as mild or suspicious neurological symptoms (e.g., vertigo, dizziness, numbness, weakness, headache, or syncope). We excluded patients who were

unwilling to undergo MRI scanning ( $n = 19$ ), for whom the detection of CMBs was missed due to MRI scanning lacking the SWI sequence ( $n = 16$ ), and who had any contraindications for MRI scanning (such as metal implants) ( $n = 12$ ). Patients with mild stroke/transient ischemic attack (TIA) ( $n = 189$ ), connective tissue disease ( $n = 4$ ), malignant disease ( $n = 7$ ), infectious disease ( $n = 23$ ), and those who had received hemodialysis ( $n = 3$ ) were excluded because such clinical diseases and conditions could influence the detection of antibodies against pathogens and inflammatory markers in the serum. Additionally, patients with a history of brain injury or craniotomy ( $n = 6$ ), intracranial occupying lesions ( $n = 8$ ), or multiple sclerosis ( $n = 5$ ) were excluded to eliminate the possible effects on the assessment of MRI images. CMBs were present in 76 of the remaining 481 patients, which were referred to as the CMBs group. Meanwhile, we randomly selected one-fifth of the remaining 405 patients who did not have CMBs as the non-CMBs group (81 patients) (Fig. 1). The principal diagnoses of the patients in the two groups were vertigo syndrome, tension-type headache, migraine, cervicogenic headache, sleep disorder, orthostatic hypotension, mild cognitive impairment, carotid arterial stenosis, intracranial arterial stenosis, and nonspecific symptoms.

The baseline characteristics of the CMBs and non-CMBs groups are shown in Table 1. Compared with the non-CMBs group, hypertension (76.3% vs. 61.7%,  $P = 0.049$ ) and the presence of lacunes (48.7% vs. 32.1%,  $P = 0.034$ ) were more frequent in the CMBs group. The CMBs group had a higher average age ( $70.1 \pm 9.7$  vs.  $66.2 \pm 10.8$ ,  $P = 0.019$ ), higher WMH grade (2 [1–3] vs. 1 [1–2],  $P = 0.019$ ), and higher serum levels of LDL ( $2.72 \pm 0.86$  vs.  $2.41 \pm 0.95$ ,  $P = 0.032$ ), CRP ( $0.79$  [0.54–1.14] vs.  $0.55$  [0.48–0.80],  $P = 0.002$ ), and three kinds of inflammatory cytokines (IFN- $\gamma$ ,  $33.67$  [26.94–44.95] vs.  $31.39$  [19.31–38.73],  $P = 0.033$ ; IL-1 $\beta$ ,  $2.12$  [1.60–3.66] vs.  $1.70$  [1.24–2.22],  $P = 0.002$ ; and IL-6,  $1.91$  [1.58–3.08] vs.  $1.73$  [1.26–2.18],  $P = 0.012$ ) than the non-CMBs group. There were no significant differences in gender, serum levels of TNF- $\alpha$ , antiplatelet/anticoagulant use, other comorbidities, and other cardiovascular risk factors between the two groups.

### Comparison of IB between CMBs and non-CMBs groups

Considering that no patient was exposed to all eight pathogens, and only a few patients were exposed to zero (10/157, 6.4%), one (17/157, 10.8%), six (12/157, 7.6%),



**Figure 1.** Selection flowchart of patients with CMBs and without CMBs. TIA, transient ischemic attack; CMBs, cerebral microbleeds.

**Table 1.** Baseline characteristics between non-CMBs group and CMBs group.

Characteristics	Non-CMBs (n = 81)	CMBs (n = 76)	P value
Male (%)	45 (55.6)	50 (65.8)	0.190
Age, y (SD)	66.2 (10.8)	70.1 (9.7)	0.019
BMI, kg/m <sup>2</sup> (SD)	23.9 (2.9)	24.0 (2.8)	0.941
Smoking history (%)	31 (38.3)	36 (47.4)	0.249
Alcohol intake history (%)	26 (32.1)	30 (39.5)	0.335
Hypertension (%)	50 (61.7)	58 (76.3)	0.049
SBP, mmHg (SD)	137.4 (19.4)	141.6 (21.8)	0.205
DBP, mmHg (SD)	83.4 (12.8)	84.3 (12.8)	0.686
Diabetes mellitus (%)	30 (37.0)	35 (48.7)	0.140
Fasting glucose, mmol/L, median, (IQR)	5.32 (4.70–8.03)	5.73 (4.81–7.74)	0.540
HbA1c, %, median, (IQR)	6.00 (5.57–7.00)	6.05 (5.50–7.48)	0.698
Hyperlipidemia (%)	35 (43.2)	43 (56.6)	0.094
LDL, mmol/L, (SD)	2.41 (0.95)	2.72 (0.86)	0.032
HDL, mmol/L, median, (IQR)	1.19 (1.04–1.43)	1.16 (0.94–1.47)	0.265
Triglycerides, mmol/L, median, (IQR)	1.24 (0.98–1.87)	1.36 (1.03–1.99)	0.492
Total cholesterol, mmol/L, (SD)	4.28 (1.19)	4.52 (1.19)	0.219
IHD (%)	5 (6.2)	4 (5.3)	0.806
Antiplatelet/anticoagulant use (%)	13 (16.0)	14 (18.4)	0.694
Presence of lacunes (%)	26 (32.1)	37 (48.7)	0.034
WMH grade, median, (IQR)	1 (1–2)	2 (1–3)	0.019
CRP, mg/L, median, (IQR)	0.55 (0.48–0.80)	0.79 (0.54–1.14)	0.002
IFN- $\gamma$ , pg/mL, median, (IQR)	31.39 (19.31–38.73)	33.67 (26.94–44.95)	0.033
	1.70 (1.24–2.22)	2.12 (1.60–3.66)	0.002

(Continued)

or seven (7/157, 4.5%) pathogens, all the patients were stratified into three categories with zero-two ( $n = 63$ ), three-four ( $n = 53$ ), and five-seven ( $n = 41$ ) seropositivities with respect to the eight pathogens analyzed. Zero-

**Table 1** Continued.

Characteristics	Non-CMBs (n = 81)	CMBs (n = 76)	P value
IL-1 $\beta$ , pg/mL, median, (IQR)			
IL-6, pg/mL, median, (IQR)	1.73 (1.26–2.18)	1.91 (1.58–3.08)	0.012
TNF- $\alpha$ , pg/mL, median, (IQR)	4.18 (2.10–5.62)	4.20 (3.23–5.96)	0.313

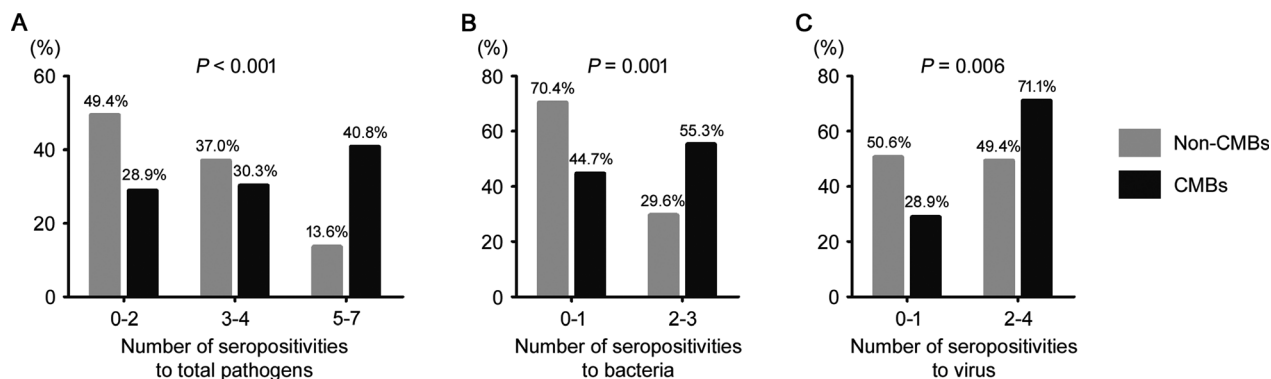
Comparison of the baseline characteristics between the two groups. Abbreviations: CMBs, cerebral microbleeds; SD, standard deviation; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; IQR, interquartile range; HbA1c, glycated hemoglobin; LDL, low-density lipoprotein; HDL, high-density lipoprotein; IHD, ischemic heart disease; WMH, white matter hyperintensity; CRP, C-reactive protein; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. Continuous variables were expressed as mean (SD) or median (interquartile range [IQR]) and compared using Independent Sample *t* test or Mann–Whitney *U* test between two groups. Categorical variables were expressed as counts (%) and Chi-square test was used for frequency comparison between two groups.

two, three-four, and five-seven seropositivities represented lower, middle, and higher IB, respectively. This classification strategy has been applied in a previous study.<sup>10</sup> As shown in Figure 2A, CMBs patients were more commonly classified in the five-seven seropositivities category (40.8% vs. 13.6%) and were less frequent in the zero-two seropositivities category (28.9% vs. 49.4%) than non-CMBs patients ( $P < 0.001$ ). There was no significant difference between the two groups in the three-four seropositivities category (30.3% vs. 37.0%).

Next, IB was classified into bacterial burden and viral burden according to a previous study.<sup>11</sup> Bacterial burden was defined as the number of seropositivities for *C. pneumoniae*, *M. pneumoniae*, HP, and *B. burgdorferi*, and was divided into two categories (zero-one and two-three) because no patient had all four bacterial seropositivities. As only a few patients had all the four viral seropositivities (21/157, 13.4%), including HSV-1, HSV-2, CMV, and EBV, viral burden was divided into two categories (zero-one and two-four). Compared with non-CMBs patients, CMBs patients were more common in the two-three seropositivities category for bacterial burden (55.3% vs. 29.6%,  $P = 0.001$ ), and the two-four seropositivities category viral burden (71.1% vs. 49.4%,  $P = 0.006$ ) (Fig. 2B and C).

### Association between single seropositivity and the presence of CMBs

The seroprevalence of each pathogen in the CMBs group tended to be higher than that in the non-CMBs group (Table 2). In univariate analysis, seropositivity toward



**Figure 2.** Percent distribution of IB in CMBs group and non-CMBs group. (A) IB consisted of 0–2, 3–4, and 5–7 seropositivities toward all the pathogens. (B) Bacterial burden consisted of 0–1 and 2–3 toward all the bacteria. (C) Viral burden consisted of 0–1 and 2–4 toward all the viruses. IB, infectious burden; CMBs, cerebral microbleeds.

HSV-1 ( $P = 0.003$ ), HSV-2 ( $P = 0.032$ ), CMV ( $P = 0.002$ ), *C. pneumoniae* ( $P = 0.001$ ), and *B. burgdorferi* ( $P = 0.010$ ) was significantly associated with the presence of CMBs. After adjusting for gender, age, hypertension, diabetes mellitus, hyperlipidemia, LDL, presence of lacunes, and WMH grade, CMV ( $P = 0.044$ , OR = 2.16, 95% CI [1.02–4.58]), *C. pneumoniae* ( $P = 0.027$ , OR = 2.32, 95% CI [1.10–4.89]), and *B. burgdorferi* ( $P = 0.040$ , OR = 2.88, 95% CI [1.05–7.92]) remained associated with the presence of CMBs.

### Association between IB and the presence of CMBs

Logistic regression analysis showed an association between IB and the presence of CMBs (Table 3). Patients in the five-seven seropositivities category were associated with the presence of CMBs when taking patients in the zero-two seropositivities category as reference ( $P < 0.001$ , OR = 4.74, 95% CI [2.01–11.21]). After adjusting for gender, age, hypertension, diabetes mellitus, hyperlipidemia, LDL, the presence of lacunes, and WMH grade, the association remained ( $P = 0.031$ , OR = 3.00, 95% CI [1.11–8.15]). Regarding the bacterial burden, when taking patients in the zero-one seropositivity category as reference, patients in the two-three seropositivities category were associated with the presence of CMBs ( $P = 0.001$ , OR = 2.93, 95% CI [1.52–5.66]), and the association remained significant after adjusting for sex, age, hypertension, diabetes mellitus, hyperlipidemia, LDL, the presence of lacunes, and WMH grade ( $P = 0.028$ , OR = 2.28, 95% CI [1.09–4.76]). With respect to the viral burden, patients in the two-four seropositivities category showed an association with the presence of CMBs when taking patients in the zero-one seropositivity category as reference ( $P = 0.006$ , OR = 2.52, 95% CI [1.30–4.87]). However,

this association did not remain after adjusting for gender, age, hypertension, diabetes mellitus, hyperlipidemia, LDL, the presence of lacunes, and WMH grade.

### Association between IB and the number and location of CMBs

In total, 314 CMBs were counted in the CMBs group. All CMBs were located in either the lobar (40.8%) or D/I region (59.2%). As the number of CMBs might be a marker of the severity of the underlying disease,<sup>3,4</sup> we investigated the association between IB and the number of CMBs. Spearman rank correlation analysis showed that IB was significantly positively associated with the number of CMBs in the total region (Spearman  $\rho = 0.653$ ,  $P < 0.001$ ) (Fig. 3A), lobar region (Spearman  $\rho = 0.607$ ,  $P < 0.001$ ) (Fig. 3B), and D/I region (Spearman  $\rho = 0.485$ ,  $P < 0.001$ ) (Fig. 3C).

In addition, according to a previous study,<sup>16</sup> the CMBs group was divided into three subgroups according to the location of CMBs: lobar (27/76 [35.5%]), D/I (31/76 [40.8%]), and mixed subgroup (CMBs were located in both lobar and D/I regions; 18/76 [23.7%]). We investigated the association between IB categories (zero-two, three-four, and five-seven seropositivities) and CMBs location. Nevertheless, the chi-square test showed that IB was not significantly associated with CMBs location ( $P = 0.071$ ) (Fig. S1).

### IB and serum inflammatory markers

We compared the levels of serum inflammatory markers among the three IB categories in the CMBs group, non-CMBs group, and total patients, respectively (Fig. 4). Compared with the patients in the zero-two seropositivities category, those in the three-four and five-seven

**Table 2.** Association between single seropositivity and the presence of CMBs

Pathogens	Frequency of seropositivity			Adjusted <sup>1</sup>	
	CMBs n (%)	Non-CMBs n (%)	Univariate P value	Multivariate P value	OR (95% CI)
HSV-1	43 (56.6%)	27 (33.3%)	0.003	0.107	1.81 (0.89–3.70)
HSV-2	44 (57.9%)	33 (40.8%)	0.032	0.426	1.34 (0.65–2.73)
CMV	48 (63.2%)	31 (38.3%)	0.002	0.044	2.16 (1.02–4.58)
<i>C. pneumoniae</i>	54 (71.1%)	37 (45.7%)	0.001	0.027	2.32 (1.10–4.89)
<i>M. pneumoniae</i>	22 (29.0%)	21 (25.9%)	0.671	0.961	1.02 (0.47–2.19)
EBV	35 (46.1%)	34 (42.0%)	0.607	0.847	1.07 (0.53–2.18)
HP	31 (40.8%)	26 (32.1%)	0.258	0.729	1.14 (0.55–2.35)
<i>B. burgdorferi</i>	18 (23.7%)	7 (8.6%)	0.010	0.040	2.88 (1.05–7.92)

Abbreviations: CMBs, cerebral microbleeds; HSV, herpes simplex virus; CMV, cytomegalovirus; *C. pneumoniae*, *Chlamydia pneumoniae*; *M. pneumoniae*, *Mycoplasma pneumoniae*; EBV, Epstein-Barr virus; HP, *Helicobacter pylori*; *B. burgdorferi*, *Borrelia burgdorferi*; OR, odds ratios; CI, confidence intervals; LDL, low-density lipoprotein; WMH, white matter hyperintensity. Univariate *P* values were derived from Chi-square test. Multivariate *P* values were derived from logistic regression analysis.

<sup>1</sup>Logistic regression analysis was adjusted for gender, age, hypertension, diabetes mellitus, Hyperlipidemia, LDL, the presence of lacunes, and WMH grade.

**Table 3.** Association between IB and the presence of CMBs

	Unadjusted		Adjusted <sup>2</sup>	
	P value	OR (95% CI)	P value	OR (95% CI)
Infectious burden				
0–2 seropositivities <sup>1</sup>		1.00 [Ref]		1.00 [Ref]
3–4 seropositivities	0.450	1.33 (0.63–2.81)	0.988	1.01 (0.44–2.30)
5–7 seropositivities	<0.001	4.74 (2.01–11.21)	0.031	3.00 (1.11–8.15)
Bacterial burden				
0–1 seropositivities <sup>1</sup>		1.00 [Ref]		1.00 [Ref]
2–3 seropositivities	0.001	2.93 (1.52–5.66)	0.028	2.28 (1.09–4.76)
Viral burden				
0–1 seropositivities <sup>1</sup>		1.00 [Ref]		1.00 [Ref]
2–4 seropositivities	0.006	2.52 (1.30–4.87)	0.155	1.70 (0.82–3.51)

Abbreviations: IB, infectious burden; CMBs, cerebral microbleeds; OR, odds ratios; CI, confidence intervals; LDL, low-density lipoprotein; WMH, white matter hyperintensity.

<sup>1</sup>Patients in this group were used as the reference.

<sup>2</sup>Logistic regression analysis was adjusted for gender, age, hypertension, diabetes mellitus, Hyperlipidemia, LDL, the presence of lacunes, and WMH grade.

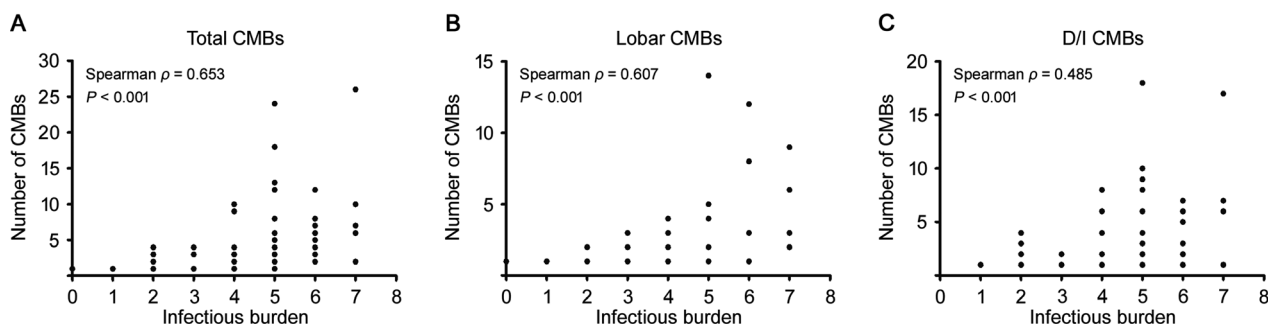
seropositivities categories had significantly higher serum levels of CRP, IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the CMBs group and total patients, respectively. Moreover, in the non-CMBs group, the serum levels of IFN- $\gamma$ , IL-1 $\beta$ ,

IL-6, and TNF- $\alpha$  in patients in the five-seven seropositivities category were significantly higher than those of the patients in the zero-two seropositivities category.

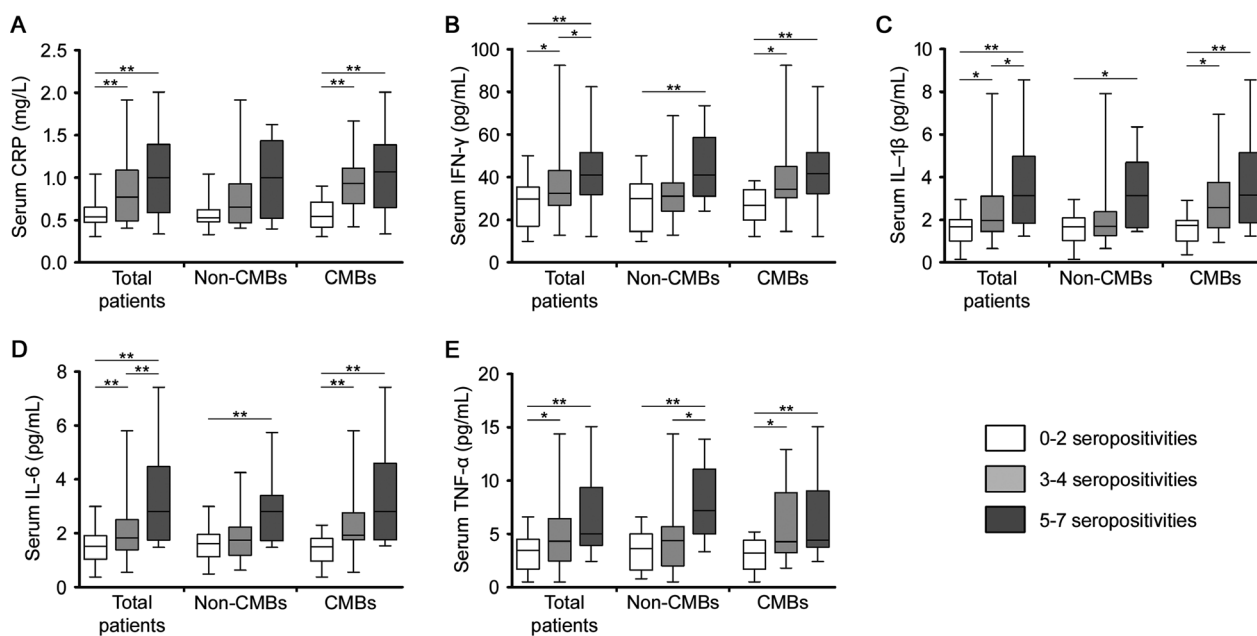
## Discussion

In this study, IB was measured by aggregating the number of single seropositivity toward HSV-1, HSV-2, CMV, *C. pneumoniae*, *M. pneumoniae*, EBV, HP, and *B. burgdorferi*. We found that: (1) IB was significantly different between the CMBs and non-CMBs groups; (2) increased IB was independently associated with the presence of CMBs; (3) IB was significantly positively associated with the number of CMBs; and (4) the levels of serum inflammatory markers were significantly different between CMBs and non-CMBs groups and among categories of IB, respectively. To our knowledge, this is the first study to investigate the association between IB and CMBs. These findings indicated that IB could be involved in the pathogenesis of CMBs.

However, the exact mechanism linking IB and CMBs is unclear. The disruption of the blood–brain barrier (BBB) could be a link between pathogen infection and CMBs. BBB disruption is probably crucial in CMBs pathogenesis. A previous study has shown that MR-visible CMBs represent focal accumulation of hemosiderin-containing macrophages in the perivascular space.<sup>17</sup> In addition, patients with CMBs have been shown to have a higher ratio of cerebrospinal fluid/serum albumin than patients without CMBs.<sup>18</sup> Moreover, the number of CMBs has been shown to be associated with increased levels of fibrin, which is a biomarker of BBB leakage.<sup>19</sup> The relationship between pathogen infection and the BBB has also



**Figure 3.** Association between IB and the number of CMBs. Analysis of association between IB and the number of CMBs in and total region (A), lobar region (B), and D/I region (C), respectively. CMBs, cerebral microbleeds; D/I, deep or infratentorial.



**Figure 4.** IB and serum inflammatory markers. Comparison of serum inflammatory markers among categories of IB in total patients, non-CMBs group, and CMBs group, respectively. Inflammatory markers included CRP (A), IFN- $\gamma$  (B), IL-1 $\beta$  (C), IL-6 (D), and TNF- $\alpha$  (E). CMBs, cerebral microbleeds; IB, infectious burden; CRP, C-reactive protein; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. \* $P < 0.05$ ; \*\* $P < 0.01$ .

been mentioned in several studies. For instance, each of the eight pathogens included in the IB in the present study were reported to be involved in the damage to BBB structure and function.<sup>20–27</sup> This was one of the reasons we selected these eight pathogens in this study.

In addition to BBB disruption, inflammation might be another link between pathogen infection and CMBs. Exposure to multiple common pathogens can induce the production of a variety of proinflammatory proteins, leading to persistent low-level inflammation,<sup>28</sup> and inflammation is closely related to CMBs. High levels of inflammatory markers, including high-sensitivity CRP, IL-6, and IL-18, are associated with CMBs in the elderly.<sup>12</sup> Blood levels of both TNF receptor 2 and myeloperoxidase

have been reported to be higher in patients with CMBs than those in patients without CMBs.<sup>29</sup> In addition, suppressing IFN- $\gamma$  can stimulate microglial response and repair microbleeds in the diabetic brain.<sup>30</sup> Moreover, lipopolysaccharide can produce a rapid and robust process of cerebral microhemorrhages in mice.<sup>31</sup>

Based on the evidence above, both BBB disruption and inflammation are probably key players in the association between pathogen infection and CMBs. Additionally, BBB disruption and inflammation have mutual effects. Inflammation can target endothelial cells and activate monocytes/macrophages, leading to the disruption of the extracellular matrix of the BBB.<sup>32</sup> Vice versa, BBB disruption can facilitate the influx of blood constituents into



the brain, which might activate microglia, recruit peripheral macrophages, and promote inflammation.<sup>33</sup>

In this study, the levels of serum inflammatory markers were significantly different between the CMBs and non-CMBs groups and among categories of IB, respectively, which was consistent with previous studies.<sup>12,15</sup> In addition, we found that seropositivity for CMV, *C. pneumoniae*, and *B. burgdorferi* was significantly associated with the presence of CMBs, which uncovered, at least in part, the association between single pathogen infection and CMBs. Several specific pathogens associated with CMBs have been reported previously. In adults with hemophilia, an infection history of hepatitis C virus has been shown to be associated with CMBs.<sup>34</sup> A population-based study has reported a strong correlation between CMBs and *Cnm* gene-positive *Streptococcus* in the oral cavity.<sup>35</sup> However, medical history and saliva samples do not represent IB; therefore, following the strategy in our previous study,<sup>15</sup> we employed the concept of IB as the aggregated number of eight common pathogens because each of these eight pathogens had been previously confirmed to be associated with stroke.<sup>36</sup>

Previous studies have revealed that an increased number of CMBs are associated with an increased risk of several neurological disorders, such as hemorrhage stroke, ischemic stroke, cognitive impairment, and dementia.<sup>3,4</sup> In this study, we found a positive association between IB and the number of CMBs, suggesting that IB might correlate with the severity of CMBs and underlying diseases. Nevertheless, we did not find a difference in IB among CMBs in different regions (lobar, D/I, and mixed), even though region-specific association between inflammation and deep CMBs have been reported.<sup>37</sup>

Cerebral small vessel disease (CSVD) is a common age-related cerebrovascular disease characterized by neuroimaging features, including small subcortical infarcts, perivascular space, brain atrophy, lacunes, WMH, and CMBs.<sup>1</sup> Several specific pathogen infections have been reported to be associated with CSVD.<sup>38</sup> However, the association between IB and CSVD remains unknown. More importantly, a previous study has shown that the remarkable degree of variation in the clinical symptoms of CSVD cannot be explained fully by a unified mechanism of vascular lesion, and routine MRI does not capture the heterogeneity present in CSVD lesions,<sup>39</sup> which led us to investigate CMBs rather than CSVD in this study.

This study has several limitations. First, we could not determine whether the serum pathogen-specific antibodies resulted from current, past, or chronic infection, although almost all previous studies used them to represent IB. Second, IB was measured by aggregating the number of

seropositivities, which did not reflect the titers and absolute concentrations of the pathogen-specific antibodies in serum. Third, this is a cross-sectional pilot study that did not determine the cause-and-effect relationship between IB and CMBs. Fourth, this study is not a large population-based study, so there might be potential bias in the sample of patients.

In conclusion, the findings of this study uncovered the association between IB and CMBs and suggested that pathogen infection could be involved in the pathogenesis of CMBs. These results need further validation in prospective studies.

## Authors' Contributions

F. F., Q. W., Y. W., and Y. X. contributed to the conception and design of the study. J. L. and R. J. contributed to the assessment of MRI images. F. F., C. Y., X. Z., Z. L., H. L., Y. Z., and X. B. contributed to the acquisition and analysis of data. F. F., C. Y., and Y. X. contributed to drafting the text and preparing the figures.

## Conflict of Interest

Nothing to report.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Association between IB and the location of CMBs.