Translational Stroke Research (2019) 10:265–272
https://doi.org/10.1007/s12975-018-0639-6

ORIGINAL ARTICLE

# High Circulatory Phosphate Level Is Associated with Cerebral Small-Vessel Diseases

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Received: 20 March 2018 / Revised: 6 June 2018 / Accepted: 12 June 2018 / Published online: 25 June 2018


## Abstract

High phosphate is linked to vascular calcification and endothelial dysfunction; however, its relationship with cerebral small-vessel diseases (CSVDs) is still unknown. Study subjects were prospectively recruited from the community-based I-Lan Longitudinal Aging Study. CSVDs including lacunes, white matter hyperintensities (WMHs), and cerebral microbleeds were evaluated using 3T magnetic resonance images. Multivariate analyses were performed to study the associations between circulatory phosphate level and the presence of CSVDs. In vitro experiments included human brain microvascular endothelial cell (HBMEC) studies and western blotting. The present study included 186 subjects (age [mean ± standard deviation, range] 64.7 ± 8.6, 50–86.8 years; 93 men). Multivariate analysis revealed that circulatory phosphate levels > 3.925 mg/dL were associated with severe WMH with an odds ratio of 3.7 (95% confidence interval = 1.3–10.6) independent of age, sex, traditional vascular risk factors, total cholesterol, renal function, or circulatory calcium level. The in vitro study revealed a downregulation of tight junction protein (zona occludens-1, occludin, and claudin-5) expression in HBMECs after 48 h of treatment with high phosphate (2.5/5 mM). We are the first to report a relationship between circulatory phosphate and CSVDs. Our results suggest that high circulatory phosphate level might be a novel risk factor for CSVD, possibly by impairing BBB structures.

**Keywords** Phosphate · Cerebral small-vessel disease · White matter hyperintensity · Blood-brain barrier · Tight junction proteins

## Introduction

Cerebral small-vessel diseases (CSVDs) refer to both pathologies affecting the perforating arterioles, capillaries, and venules, which lead to tissue damage mainly in the white matter, and neuroimaging abnormalities, including lacunes, white matter hyperintensities (WMHs), and cerebral microbleeds (CMBs) [1–3]. Clinically, they are associated with neuropsychological impairment, gait disturbance, and physical frailty and also predict a higher risk for dementia, disability, and mortality [2–7]. Though their clinical significances are certain, their pathogenesis remains under study [1–11]. Several clinical studies have shown that an elevated level of circulatory phosphate is associated with mortality and cardiovascular events including coronary heart disease and stroke, not only in patients with chronic kidney disease (CKD) but

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also in individuals with normal renal functions [12–16]. Nevertheless, the relationships between circulatory phosphate and CSVDs have not yet been studied. We aimed to investigate the associations between high circulatory phosphate and (1) CSVDs including lacunes, WMHs, and CMBs in a community-based general population and (2) the expression of tight junction proteins, which are components of the blood-brain barrier (BBB) [17, 18], in human brain microvascular endothelial cells (HBMECs). We hypothesized that high phosphate might be a novel risk factor for CSVDs and influence the expression of tight junction proteins in HBMECs.

## Materials and Methods

### I-Lan Longitudinal Aging Study and the General Assessment

I-Lan Longitudinal Aging Study (ILAS) was an ongoing community-based cohort study conducted in I-Lan County, Taiwan. The study protocol has been previously described in detail [19]. Inhabitants who met the study inclusion criteria were randomly sampled from the household registration data of the county government. Selected inhabitants were invited to participate by mail or telephone. The inclusion criteria were (1) having no plans to move out of I-Lan County in the near future and (2) being 50 years or older. Subjects who met any one of the following conditions were excluded: (1) unable to adequately communicate with the interviewer, (2) having a disability (modified Rankin Scale score > 2), (3) limited life expectancy (less than 6 months) due to major illness, and (4) currently institutionalized. A questionnaire was used to collect data regarding the demographics, years of education, smoking or/and alcohol consumption habits, and medical histories of the subjects. Alcohol drinking was defined as more than 4 drinks on any single day or more than 14 drinks per week for men and more than 3 drinks on any single day or more than 7 drinks per week for women. The heights, weights, and resting blood pressures (BPs) of the subjects were measured. All participants underwent a face-to-face neuropsychological examination administered by trained interviewers. Global cognitive performance was assessed using the Mini-Mental State Examination (MMSE) [20, 21]. Global cognitive impairment was defined as an MMSE score < 24 in well-educated subjects (education years ≧ 6) or an MMSE score < 14 in less-educated subjects (education years < 6) [21].

Cardiovascular risk factors were either measured or assessed by self-report. Hypertension was defined as a self-report of current antihypertensive medication prescription or a measurement of systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mmHg. Diabetes mellitus (DM) was defined as a self-report of current DM medication use or a measurement of hemoglobin A1c (HgbA1c) ≥ 6.5%. Hyperlipidemia was defined as a self-report of the use of statins or a total cholesterol blood level ≥ 240 mg/dL. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meter. CKD was defined as an estimated glomerular filtration rate (eGFR) ≤ 60 mL/min/1.73 $m^2$ [22].

### Study Population for the Present Study

The study population of the present study comprised a subgroup of the ILAS cohort prospectively recruited from September 2014 to July 2015. To evaluate the effects of circulatory phosphate on CSVDs, which are difficult to validate when there are other confounding factors, we excluded subjects with CKD, stroke history, or global cognitive impairment. The study had been approved by the Institutional Review Board of National Yang Ming University, and all participants had signed informed consents.

### Circulatory Phosphate Levels

Fasting morning peripheral blood was drawn and serum was isolated from all subjects. The levels of phosphate, calcium, intact parathyroid hormone (iPTH), 25-hydroxyvitamin D (VitD), sugar, HgbA1c, lipid (total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglycerides), blood urea nitrogen, and creatinine were determined using a chemical analyzer (ADVIA 1900, Siemens; Malvern, PA, USA).

#### Brain Magnetic Resonance Image Acquisition

All participants underwent baseline brain magnetic resonance image (MRI) at National Yang-Ming University within 1 month of the neuropsychological assessment and serum collection. Images were acquired on a 3T MRI scanner (Siemens Magnetom Tim Trio; Erlangen, Germany) with a 12-channel head coil. An axial T2-weighted fluid attenuated inversion recovery (FLAIR) multi-shot turbo spin echo sequence with the BLADE technique was acquired using the following parameters: repetition time (TR) = 9000 ms, echo time (TE) = 143 ms, inversion time = 2500 ms, flip angle = 130°, number of excitations = 1, echo train length = 35, matrix size = 320 × 320, field of view (FOV) = 220 × 220 $mm^2$, 63 slices, bandwidth = 252 Hz/Px, voxel size = 0.69 × 0.69 × 2.0 $mm^3$ without inter-slice gap, and acquisition time = 7 min and 41 s. Three-dimensional susceptibility-weighted images (SWIs) with the following parameters were used to identify CMBs: TR = 28 ms, TE = 21 ms, flip angle = 15°, matrix size = 256 × 224, FOV = 256 × 224 $mm^2$, 88 slices, bandwidth = 120 Hz/Px, voxel size = 1.0 × 1.0 × 2.0 $mm^3$ without inter-slice gap, and acquisition time = 9 min and 13 s.

## CSVD Assessment

All images were displayed and viewed using MRIcro software (version 1.40, Chris Rorden's MRIcro) by the same neurologist (Dr. Chung), who was blind to the subjects' clinical data during the imaging assessment. The presence of lacunes and WMHs was recorded and the lacunes and WMHs were rated based on FLAIR T2-weighted MR images. Lacunes are small cerebrospinal fluid-containing cavities smaller than 15 mm in diameter and are located in deep gray or white matter with adjacent WMH [3]. WMH severity was rated using the modified Fazekas scale [23], wherein a score of 0 indicates no WMH and scores of 1 to 3 indicate increasing extents of WMH. We defined severe WMH based on Fazekas scores of 2 or 3. CMBs were defined as small rounded or circular well-defined hypointense lesions within brain parenchyma with clear margins and sizes ≦ 10 mm on SWI images [3]. Microbleed mimics, such as vessels, calcification, partial volumes, air-bone interfaces, and hemorrhages within or adjacent to an infarct were carefully excluded. We used the Microbleed Anatomical Rating Scale [24] to assess the presence, amounts, and topographic distributions of CMBs in each subject. Microbleeds were categorized as deep, lobar, or infratentorial. Lobar topography was determined according to the method established by Stark and Bradley [25] in cortical and subcortical regions (including subcortical U fibers). Lobar CMBs were assessed in the frontal, parietal, temporal, and occipital regions. Deep regions included the basal ganglia, thalamus, internal capsule, external capsule, corpus callosum, and deep/periventricular white matter. Infratentorial regions included the brainstem and the cerebellum.

## Cell Culture and Western Blotting

Commercialized HBMECs were obtained from three different sources (cat. no. ACBRI376, Cell Systems; Kirkland, WA, USA). The number ($n$) corresponds to the total number of repeated experiments performed collectively using cells from all three sources. HBMECs were treated with EC CSC medium (cat. no. 4Z3-500, Cell Systems) containing 5% fetal bovine serum. Dulbecco's Modified Eagle Medium was supplemented with β-glycerolphosphate disodium (cat. no. G9422, Sigma; St. Louis, MO, USA) for the phosphate experiments. The cells used in this study were at passage 8 or earlier. HBMECs were assessed regularly to confirm their central nervous system properties. The cells were stained with antibodies against von Willebrand factor to confirm their EC origins. The cultures were analyzed routinely for astrocyte contamination by staining with anti-glial fibrillary acidic protein.

Sample media was mixed with 4× loading (sample) buffer containing 5% β-mercaptoethanol (Sigma) and radioimmunoprecipitation assay buffer, pH 7.4 (cat. no. BP-115, Boston BioProducts; Ashland, MA, USA). The samples were then heated for 5 min at 95 °C. We loaded 10–30 μg of sample protein onto NuPAGE Bis-Tris precast polyacrylamide gels (each lane was loaded with the same amount of protein within a Western blot) and performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the mini-cell system (Invitrogen; Carlsbad, CA, USA) and NuPAGE 3-(N-morpholino)propanesulfonic acid SDS running buffer (Invitrogen) was used. We added 500 μl of antioxidant to the running buffer. Electrophoresis was performed at 140–200 V until adequate spread of the protein molecular marker was achieved. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes (Millipore; Burlington, MA, USA). Transfer was achieved using a wet blot (Bio-Rad; Hercules, CA, USA) transfer system. We used standard Towbin transfer buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine, and 20% ($v/v$) methanol. Proteins were then visualized using an enhanced chemiluminescence detection system.

All primary antibodies used for western blotting were prepared in Tris-buffered saline containing 0.2% Tween-20. The antibodies were used at the following dilutions: anti-zona occludens-1 (Zo-1) (cat. no. 33-9100, Invitrogen) at 1:500, anti-occludin (cat. no. 33-1500, Invitrogen) at 1:500, and anti-claudin-5 (cat. no. 35-2500, Invitrogen) at 1:500.

## Statistical Analyses

Analyses were performed using SPSS software (version 22.0, IBM, Armonk, NY, USA). All data are presented as mean (standard deviation [SD]) for continuous variables and number (percentage) for discrete variables. We classified the study population into quartiles according to the level of circulatory phosphate. Group comparisons were made using two-tailed paired $t$ tests (the top quartile versus those in the lower three quartiles) or one-way analyses of variance (four quartile groups) followed by Bonferroni *post hoc* analysis. When appropriate, a chi-square ($\chi^2$) test or Fisher's exact test was performed for categorical variables. Multivariate logistic regression analyses were used to test the association between high circulatory phosphate (the top quartile) and the presence and severity of CSVDs. Confounding factors, such as age, sex, vascular risk factors (hypertension, DM, smoking, alcohol consumption, BMI, total cholesterol), eGFR, and level of circulatory calcium were adjusted. The results are presented as odds ratios (ORs) with 95% confidence intervals (95% CIs).

## Results

### Characteristics of Subjects with Different Levels of Circulatory Phosphate

One-hundred and eighty-six subjects (age [mean ± SD, range] 64.7 ± 8.6, 50–86.8 years; 93 men) were included in the present study. The level of circulatory phosphate in the subjects was 3.6 ± 0.5, 2.3–5.0 mg/dL. We classified the study population into quartiles according to the level of circulatory phosphate. The clinical characteristics of the subjects are shown in Table 1. There was no difference in the vascular risk factor profile among the four groups, with the exception of the level of circulatory total cholesterol (TC). *Post hoc* Bonferroni analyses revealed that the levels of TC were significantly lower in the 3rd quartile than in the 1st quartile. We also measured parameters associated with both phosphate homeostasis and vascular health, including calcium, iPTH, and VitD levels [26]. Circulatory calcium levels were significantly lower in the 2nd quartile than in the 3rd and 4th quartiles, as determined using *post hoc* Bonferroni analyses. There were no differences in the levels of PTH and VitD among the four groups.

### High Circulatory Phosphate Is Associated with Severe WMH

In the present study population, 30 (16.1%) subjects had severe WMHs, 24 (12.9%) had at least one lacune, and 12 (6.5%) and 10 (5.4%) had at least one CMB in the deep or infratentorial and lobar regions, respectively. Table 2 shows the prevalences of and comparisons between CSVDs in subjects in the top quartile versus those in the lower three quartiles according to phosphate level. The subjects in the top quartile of circulatory phosphate had a significantly higher prevalence of severe WMH than those in the lower circulatory phosphate quartiles. High circulatory phosphate was not associated with other CSVDs, lacunes, or CMBs in our population.

We performed multivariate analyses to evaluate the association between high circulatory phosphate and severe WMH (Table 3). Circulatory phosphate levels > 3.925 mg/dL were associated with severe WMH with an OR of 3.7 (95% CI = 1.3–10.6, $P = 0.014$). This association was independent of age, sex, traditional vascular risk factors, total cholesterol, renal function (eGFR), or circulatory calcium level.

### High Phosphate Downregulates the Expression of Tight Junction Proteins in HBMECs

Treatment of ECs with phosphate has been shown to lead to such effects as induction of apoptosis, decreased nitric oxide (NO) production, senescence, induction of microparticle shedding, and inhibition of angiogenesis [16, 27, 28]. However, its effect on the BBB, one characteristic of brain microvascular EC has not been studied yet. It is postulated that increased permeability due to BBB impairment might be involved at the early stages of WMH development [1, 29]. We tested whether high phosphate levels affect BBB structures by evaluating the expression levels of tight junction proteins in HBMECs in a high-phosphate milieu. Western blotting revealed a dose-dependent trend for the downregulation of tight junction proteins in HBMECs after 48 h of phosphate treatment (Fig. 1). There was significant downregulation of occludin expression following treatment with 1 mM phosphate. The expression levels of the three tight junction proteins Zo-1, occludin, and claudin-5 were all significantly decreased in response to treatment with higher levels of phosphate (2.5 and 5 mM) when compared with control treatment.

## Discussion

We are the first to report a relationship between circulatory phosphate levels and CSVDs. First, in people older than 50 years who had no stroke, dementia, or CKD, higher circulatory phosphate levels were associated with severe WMH. This effect was independent of age, sex, or traditional vascular risk factors. Second, the in vitro cellular study revealed a downregulation of the tight junction proteins Zo-1, occludin, and claudin-5 in HBMECs after treatment with high phosphate levels. Our results suggest that high circulatory phosphate might be a novel risk factor for CSVD and may act by impairing BBB structures.

The prevalence and severity of CSVDs, particularly WMHs, is higher in patients with CKD [30–36]. This relationship has been shown to be independent of traditional vascular risk factors, including age and hypertension. These brain lesions are presumed to be responsible for the frequently observed cognitive impairment in patients with CKD [30]. The mechanism underlying CKD-related CSVD pathogenesis is not certain and might be multifactorial. For instance, it may involve uremic toxins, chronic inflammation, or oxidase stress [30–36]. Hyperphosphatemia is a main feature of CKD, and the level of circulatory phosphate increases proportionally with reductions in the GFR in patients with CKD [37, 38]. Although hyperphosphatemia has been known as a major risk factor for cardiovascular events and mortality in patients with CKD [14–16], there is insufficient information regarding the relationship between hyperphosphatemia and CSVD in these patients. Our findings may help elucidate the pathogenesis of CKD-related CSVD. Here, we provide evidence that a high circulatory phosphate level may cause cerebral microvascular abnormalities. Thus, a high circulatory phosphate level might be involved in the pathophysiology of CKD-related CSVD.

The prevalence and severity of WMH significantly increase with aging and are related to age-related neuropsychological

**Table 1** Characteristics by circulatory phosphate quartiles

| Phosphate (mg/dL) | Total population = 186 | | | | *p* |
|---|---|---|---|---|---|
| | 1st quartile (≤ 3.300) | 2nd quartile (3.301–3.600) | 3rd quartile (3.601–3.925) | 4th quartile (> 3.925) | |
| Age, years | 64.5 (8.2) | 66.4 (9.8) | 62.7 (8.2) | 65.1 (8.2) | 0.295 |
| Sex, man, *n* | 33 (57.9%) | 17 (41.5%) | 20 (47.6%) | 23 (50.0%) | 0.438 |
| HTN, *n* | 17 (29.8%) | 21 (51.2%) | 18 (42.9%) | 14 (30.4%) | 0.102 |
| DM, *n* | 7 (12.3%) | 8 (19.5%) | 7 (16.7%) | 6 (13.0%) | 0.750 |
| Dyslipidemia, *n* | 3 (5.3%) | 4 (9.8%) | 6 (14.3%) | 4 (8.7%) | 0.494 |
| Smoking, *n* | 19 (33.3%) | 14 (34.1%) | 15 (35.7%) | 19 (41.3%) | 0.694 |
| Alcohol consumption, *n* | 28 (49.1%) | 15 (36.6%) | 22 (52.3%) | 28 (60.9%) | 0.199 |
| SBP, mmHg | 127.3 (14.8) | 129.4 (12.7) | 133.3 (20.0) | 130.0 (17.5) | 0.469 |
| DBP, mmHg | 76.6 (10.2) | 78.9 (10.5) | 80.5 (14.4) | 79.9 (13.9) | 0.485 |
| HgbA1C, % | 6.2 (1.1) | 5.9 (0.6) | 6.2 (1.2) | 6.0 (0.8) | 0.666 |
| TC, mg/dL | 203.6 (39.4) | 187.8 (32.1) | 181.5 (28.4) | 194.3 (35.8) | 0.036 |
| HDL, mg/dL | 54.3 (13.1) | 54.4 (13.7) | 53.6 (14.2) | 53.4 (11.5) | 0.983 |
| LDL, mg/dL | 124.7 (37.0) | 113.1 (29.7) | 107.2 (29.8) | 123.2 (37.0) | 0.078 |
| TG, mg/dL | 126.0 (59.6) | 102.4 (44.1) | 111.6 (64.5) | 102.3 (51.9) | 0.090 |
| BMI, kg/m$^2$ | 24.6 (3.0) | 24.4 (3.8) | 25.2 (3.7) | 24.0 (3.3) | 0.384 |
| eGFR, mL/min/1.73m$^2$ | 89.3 (18.3) | 93.9 (24.3) | 96.7 (26.3) | 94.2 (20.9) | 0.501 |
| Phosphate, mg/dL | 3.0 (0.3) | 3.5 (0.1) | 3.8 (0.1) | 4.2 (0.2) | – |
| Calcium, mg/dL | 9.1 (0.5) | 8.9 (0.3) | 9.2 (0.4) | 9.2 (0.5) | 0.002 |
| Albumin, g/dL | 4.5 (0.2) | 4.5 (0.2) | 4.5 (0.2) | 4.5 (0.2) | 0.307 |
| iPTH, ng/mL | 38.8 (16.8) | 41.6 (14.9) | 35.5 (19.0) | 40.7 (15.7) | 0.138 |
| VitD, ng/mL | 26.1 (7.7) | 23.8 (6.6) | 24.6 (5.0) | 23.5 (5.5) | 0.356 |

Presented as means (SD) or numbers (percentages)

*HTN* hypertension, *DM* diabetes mellitus, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *TC* total cholesterol, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *TG* triglyceride, *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *iPTH* intact parathyroid hormone, *VitD* 25-hydroxyvitamin D

functional impairment [1–3]. However, the etiology of WMH is largely unknown. Studies have shown that vascular risk factors such as hypertension, DM, dyslipidemia, and smoking are only partly accountable for the presence of CSVDs [11, 39]. A large community-based study of the elderly has shown that while traditional vascular risk factors explain 70% of large artery atherosclerotic disease, only 1.4–2% of variance in WMH incidence is explained by these factors [11]. Clinical trials with antihypertensive medications or statins have also failed to prevent WMH progression [39–42]. Thus, investigations of the mechanisms underlying age-related WMHs other than those related to traditional vascular risk factors are required. In addition to renal function impairment, age is associated with higher circulatory phosphate levels [12, 13]. Both the Framingham Offspring and Atherosclerosis Research in Community community-based studies, which involved large populations, have shown that serum phosphate values are positively and independently correlated with age [12, 13]. Here, we found that a high circulatory phosphate level is independently related to severe WMH. This indicates that a high circulatory phosphate level might be a novel risk factor for age-related WMH. This relationship was observed in relatively healthy subjects without a history of stroke or dementia older than 50 years. This suggests that the potential involvement of circulatory phosphate in WMH might be important at earlier disease stages.

**Table 2** Cerebral small-vessel disease in the top and the lower three quartiles of phosphate

| Phosphate level | Top quartile | The lower three quartiles | *p* |
|---|---|---|---|
| Number | 46 | 140 | |
| Severe WMH, *n* | 12 (26.1%) | 18 (12.9%) | 0.034 |
| Lacune, *n* | 6 (13.0%) | 18 (12.9%) | 0.974 |
| CMB, *n* | | | |
| Deep/infra | 3 (6.5%) | 9 (6.4%) | 0.982 |
| Lobar | 3 (6.5%) | 7 (5.0%) | 0.691 |

Presented as numbers (percentages)

*WMH* white matter hyperintensity, *CMB* cerebral microbleed, *Deep/infra* deep or infratentorial regions

As mentioned above, several studies have attempted to elucidate the pathogenesis of WMH without complete

**Table 3** Multivariate-adjusted associations between high circulatory phosphate and severe white matter hyperintensity

| Severe white matter hyperintensity | OR | 95% CI | *p* |
|---|---|---|---|
| High circulatory phosphate (> 3.925 mg/dL) | | | |
| Adjusted with age, sex | 2.8 | 1.1–7.3 | 0.029 |
| Adjusted with age, sex, HTN, DM, smoking, alcohol consumption, BMI, and total cholesterol | 3.6 | 1.3–10.0 | 0.014 |
| Adjusted with age, sex, HTN, DM, smoking, alcohol consumption, BMI, total cholesterol, and eGFR | 3.6 | 1.3–10.2 | 0.014 |
| Adjusted with age, sex, HTN, DM, smoking, alcohol consumption, BMI, total cholesterol, eGFR, and calcium | 3.7 | 1.3–10.6 | 0.014 |

*HTN* hypertension, *DM* diabetes mellitus, *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *OR* odds ratio, *CI* confidence interval

success. Neuroimaging studies have provided insight into WMH pathophysiology at earlier disease stages when compared to pathological studies [1, 43, 44]. These studies have led to the hypothesis that impaired endothelial BBB function leading to increased permeability might occur in patients with WMH before the development of microvascular occlusion and consequent brain tissue ischemia due to typical microvascular wall pathology (e.g., lipohyalinosis and collagen arteriosclerosis). We found a downregulation of tight junction proteins in HBMECs after high phosphate treatment, providing evidence that high phosphate levels might impair BBB structures and functions and lead to WMH. Whether a high phosphate level causes BBB dysfunction warrants further studies, such as in vitro and in vivo BBB permeability assays, as well as clinical neuroimaging evaluations.

Other potential mechanisms may explain the relationship between high circulatory phosphate levels and WMH. Though few researchers have studied the effects of circulatory phosphate on cerebral microvessels, many have focused on medium- to large-sized arteries. These researchers have reported the involvement of high phosphate levels in vascular calcification and stiffness [14, 16, 45, 46]. Given that aorta stiffness is associated with CSVDs in large community-based studies [47, 48], high circulatory phosphate levels might cause CSVDs via this mechanism. In addition to the effects of high phosphate levels on vascular smooth muscle cells, clinical and in vitro studies have reported endothelial dysfunctions, such as induction of apoptosis, decreased NO production, senescence, induction of microparticle shedding, and inhibition of angiogenesis under high-phosphate conditions [16, 27, 28]. Whether high phosphate has similar effects on brain microvascular endothelial cells and consequently leads to CSVDs would also require further studies. Lastly, higher serum phosphorus levels may indicate subclinical renal dysfunction, with its associated cerebral microvascular damage.

Phosphate in our body is mainly from dietary intake and the content in human body is regulated by three hormones, PTH, VitD, and fibroblast growth factor 23 (FGF23) that act on the intestine, skeleton, and kidneys [46]. Of these, the kidney is the major site for phosphate excretion and minute-to-minute regulation of phosphate homeostasis; approximately 70% of the filtered phosphate is reabsorbed within the proximal tubule. High PTH levels, as in hyperparathyroidism, lead to renal phosphate wasting and hypophosphatemia, while low PTH levels, as in hypoparathyroidism, lead to increased renal phosphate reabsoption and hyperphosphatemia. Similar

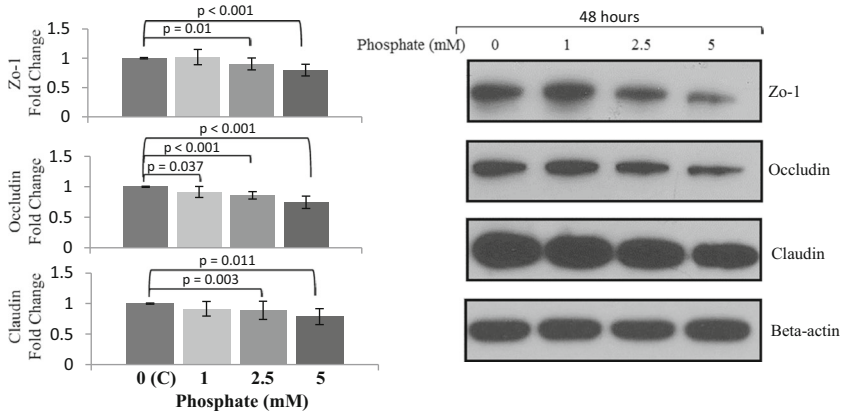


**Fig. 1** The expression of tight junction proteins after phosphate treatment. High phosphate downregulates the expression of tight junction proteins, Zo-1, Occludin, and Claudin, in human brain microvascular endothelial cells (HBMECs). Phosphate treatment decreased the expression of tight junction proteins in a dose-dependent manner in HBMECs. The Zo-1, Occludin, and Claudin protein downregulation following stimulation with 2.5 and 5 mM for 48 h was detected by Western blot; $n = 6$. Quantitative analysis of Western blot by densitometry is normalized to actin. Data represent mean ± SD of at least three independent experiments. *P* value was obtained by ANOVA test with Bonferroni post hoc analysis

to PTH, FGF23 suppresses phosphate reabsorption in the proximal tubule. However, PTH and FGF23 have opposite effects on VitD production. PTH increases and FGF23 decreases the proximal renal tubular expression of 25-hydroxyvitamin D 1a-hydroxylase that catalyzes the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (VitD). VitD in turn regulates circulatory phosphate concentration by increasing intestinal calcium and phosphate absorption [46]. Thus, higher circulatory phosphate level might be attributed by increased oral intake, or subclinical renal or related-hormone homeostasis impairment in our cases.

We measured variables associated with phosphate homeostasis, such as serum calcium, iPTH, and VitD levels in our population and excluded their possible confounding effects on the presence of CSVDs [26]. The other strengths of our study included the community-based study population comprising individuals who were relatively healthy and had no stroke, cognitive impairment, or CKD. This is indicative of an independent association between circulatory phosphate levels and CSVDs. In addition, we measured fasting morning phosphate levels in all subjects to avoid potential circadian variations in circulatory phosphate levels [46]. Finally, we used 3T MRI scanning, which has good resolution for the study of CSVDs, and SWI, a sequence that has high sensitivity for CMB detection [49].

There are limitations of the present study. First, owing to the small population with CMBs and lacunes in this study, we were unable to conclude whether circulatory phosphate is associated with these CSVDs. Second, the study was conducted in an Asian population; therefore, it is unclear if the results obtained extend to Western populations and will thus require further investigation. Third, we did not assess the clinical significance of the CSVDs using tools such as neuropsychological tests in the subjects with high circulatory phosphate levels. Fourth, we did not perform a BBB functional study which would need co-culture with the other cells such as astrocyte or/and pericytes [17, 18] to validate our hypothesis that high phosphate levels might cause CSVDs by impairing the BBB integrity. Lastly, as the present study had a cross-sectional design, we were unable to determine whether the correlation between high circulatory phosphate levels and WMH was a causal relationship.

In conclusion, we found an independent association between high circulatory phosphate levels and severe WMH in a relatively healthy population and a downregulation of tight junction proteins in HBMECs. High circulatory phosphate might be a potential treatment target for CSVDs. Further clinical longitudinal studies with larger populations, as well as basic studies using other BBB functional assays, are required to validate whether and how high circulatory phosphate levels cause CSVDs. As to the potential interventions for CSVD prevention, decreased oral phosphate intake with designed diet formula or/and phosphate binder to decrease phosphate content in human body may be evaluated in the future clinical studies.

**Funding** This study was funded by Taiwan Ministry of Science and Technology, and Taipei Veterans General Hospital, Taiwan (Chung:VGH V105C-055; V107C-048; MOST 104-2314-B-075-MY3; Chen:MOST103-2633-B-400-002; MOST105-3011-B-010-001; Veterans Affair Council of Taiwan 105-X2-2-1).

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (The Institutional Review Board of National Yang Ming University approved the present study) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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